Understanding the Biology and Epidemiology of *Dickeya solani*: a Recently Emerged Bacterial Pathogen of Potato

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&

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Abstract

Dickeya solani has emerged as a major threat to potato production in Europe and Israel. Its potential impact on the Scottish potato industry was studied with the goal to develop better control strategies. Molecular techniques were employed to understand the diversity of the *D. solani* and its comparison to other *Dickeya* spp. and to map the origin and spread of infection. Using nine housekeeping genes, a MLSA typing scheme was created and published online to facilitate its future tracking. MLSA established genetic variation between *D. solani* isolates and it was determined that *D. solani* is likely to be clonal. Due to the similarities between *D. solani* isolates, which was further aided with the sequencing of three full *D. solani* genomes, single nucleotide polymorphisms were discovered. Eight SNPs were identified and further investigated; allowing for the development of pyrosequencing assays.

Two real-time PCR assays were developed to improve the diagnostics of *D. solani* by detecting and identifying *D. solani* specifically. These assays were further evaluated as part of the Euphresco II project on blackleg and soft rot disease. The spread of *D. solani* is likely to be through latently infected seed potatoes; therefore, the transmission of *D. solani* from infected seed potato to daughter tubers was studied. It has also been suggested that *D. solani* can survive and spread through stored tubers and the potential for *D. solani* to survive in plant material was assessed. The effectiveness of disinfectants commonly used in agriculture was also investigated and it was determined when used at the manufacturer's recommended concentration, they were effective at controlling the pathogen on surfaces.

Dedicated to Kelly family:

Mary, Shaun, Natalie, Lauren and Timothy.

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Chapter 1: Introduction

1.1 The History of Potato

The *Solanaceae* family comprises of over 300 genera and contains the genus *Solanum* which, in turn, contains over one thousand species, many of which are used for both food and medicinal purposes and include tomato, pepper, aubergine and potato (Knapp *et al.*, 2004). The cultivated potato, *Solanum tuberosum*, originates from South America, specifically in the Andes, on the border between Bolivia and Peru (Spooner *et al.*, 2005). Records of human consumption of potatoes date back 8,000 years and archaeological evidence of the consumption of potatoes, particularly in the region of the Andes Mountains, have been found from 200 A.D. with images of potatoes found on pottery, indicating the importance of the crop in early Peruvian culture and society (Salaman & Burton, 1985). It has been suggested that the development of civilisations in the Andes was greatly influenced by the potato and the high level of social development was based on the energy found in the potato which had become their staple food (Glave, 2001).

The chemical composition of potato has been characterised for some cultivars and reflects its importance as a food crop. Approximately 75% of its total dry matter is carbohydrate, and it is also a good source of protein, vitamins, dietary fibre and certain minerals (Storey, 2007). An average serving of potato (175g) provides over 40% of the recommended daily allowance for vitamin C, approximately 30% of vitamin B6 requirement, 16% of vitamin B1, 16% of folate requirement and is also an important dietary source of potassium (18%), iron (6%) and magnesium (Storey, 2007).

The potato was introduced to Europe in the 1570s, after the conquest of the Inca Empire by Francisco Pizarro and his subsequent return to Spain (Harding, 1993). Initially, the potato was primarily cultivated as feed for livestock by farmers, as Europeans were sceptical of using it as a food source; however, in the 17th century, global distribution of the potato began and it now ranks globally behind rice and wheat as the third most important food crop (Chapman, 2000; Reader, 2008; FAO, 2012). Potatoes are now grown in 149 countries and grown in latitudes ranging from 65^{o}N to 50^{o}S and altitudes ranging from sea level to 4,000 metres, demonstrating the versatility and adaptability of the potato to many environmental conditions (Birch *et al.*, 2012). Potato production has increased by 21% overall in the period from 1991 to 2007 with approximately 50% of the potatoes consumed fresh and much of the remaining half processed into food

products and ingredients, animal feed or used as seed tubers for the following season's potato crop (FAO, 2012; Birch *et al.*, 2012).

1.2 The Development of the Cultivated Potato

It is believed that *Solanum tuberosum* was originally derived from the hybridisation of closely related species of *Solanum* including the *S. brevicaule* group (*S. bukasovii, S. candolleanum*) as well as *S. avilesii, S. berthaultii, S. infundibuliforme, S. oplocense, S. sparsopilum, S. sucrense* and *S. verrucosum*; however cultivated potatoes only represent a small fragment of the genetic diversity of potato (Rodrigues *et al.*, 2010). With the continual development of improved and higher resolution genotyping technologies, the taxonomy of wild species is constantly in flux with the number of recognised wild species changing from 217 to 100 (Hawkes, 1990; Hijmans and Spooner, 2001; Rodriguez *et al.*, 2010). Wild potatoes have adapted to a wide range of habitats and climates, and climate change could have significant impact on the diversity of wild species with recent studies estimating that more than 10% of the known potato species face extinction (Hijmans and Spooner, 2001; Jarvis *et al.*, 2008).

One of the most important, but uncontrollable factors, affecting the growth and yield of potato is temperature. Heat stress has significant impact on global agriculture and food security, and it is essential that crops are able to adapt adequately to these stressors (Battisti and Naylor, 2009). In many potato genotypes, tuber yield is highly responsive to elevated temperatures, with optimal temperatures for tuber yield ranging between 14^{o}C to 22^{o}C, and at temperatures above this, yield significantly decreased (Van Dam *et al.*, 1996). The effect of elevated temperature is thought to impact multiple physiological processes in the potato plant and tuber development, with tuberisation signalling inhibited at higher temperatures (Ewing, 1981). Global warming is predicted to result in overall decreased global yields ranging from 10-29% in the time period from 2010-2039 and up to 18-31% decrease in 2050s (Hijmans, 2003). To combat the effects of temperature change due to global warming, adaptive measures can be implemented such as shifting of the planting time (by one or two months) and using cultivars that are adapted to have a later foliage senescence. Using preventative measures will still result in decreased yields, however, this is estimated to be approximately 40% less than without the use of such measures (Birch *et al.*, 2012). Global warming will have a more significant impact in Asia and Sub-Saharan Africa where there is less scope for adaptations in comparison to those countries in Northern regions.

1.3 The Significance of Potato as a Crop

The potato is an important crop in terms of food security, with over one billion consumers globally, and as the global population continues to grow so will hunger rates. Over the next 20 years, it is expected that the potato will contribute to 50% of the increased food production in China, the world's biggest consumer of potato, to meet the country growing food demands (Jansky *et al.*, 2009). Overall potato production has increased by 21% from 1991 to 2007; with production in the developing world increasing by 48%, however, the production in the developed world has decreased by 12% (FAO, 2012). From the period of 1992 to 2010 Europe was the major producer of potatoes, accounting for 44.5% of the global harvest, and Asia ranked second with 37.5% of the production; however, since 2010, potato production in Europe has decreased to 33.3%, whilst increasing in Asia, where production was 47.5% of the total global potato production (FAO, 2012). The five highest potato producers have been China, The Russian Federation, India, the USA and Poland; however, production in China and India have increased dramatically, accounting for the increase seen overall in Asia, whilst production in the Russian Federation, the USA and Poland has steadily decreased (FAO, 2012). Worldwide, an estimated 19 million hectares are devoted to potato production, with an average yield of 17 tonnes per hectare (FAO, 2012). In Europe, a reduction in the area devoted to the crop accounts for the decline in potato production rather than a reduction in yield, whereas in Asia, an increase in the area devoted to potato production can account for the increase seen (FAO, 2012). Potato yields vary from country to country, but overall, seven of the ten highest producing countries are in Northern Europe, and the lowest three are in Africa (FAO, 2012). Potato has traditionally been a cash crop in Sub-Saharan Africa, and its growth is becoming increasingly important; consequently, the area used for growing potato, the production and yield has increased from 2007 to 2010.

International trade of potato has increased over the past 30 years, although the crop is regarded as bulky and perishable and transportation costs are high, with exports doubling from 1985 to 2009 (FAO, 2012). Increased demand from the fast food industry for frozen and processed products has driven this growth and the increase in trade has been largest in the developed world, with Europe being both the main exporter and importer of potato (FAO, 2012). Overall, international trade remains a small proportion of potato production primarily due to the costs of transportation and the high tariffs

placed on processed potato in order to protect the local processing industry which limits the trade in the developing world (Birch *et al*., 2012).

The potato is regarded as one of the most important global crops due to its use in human consumption and its use in the starch industry (Fabeiro *et al*., 2001). Potato starch possesses many unique features compared to starch obtained from cereals, its smooth granules allows it to be used in the manufacture of paper and the generation of viscous hydrocolloid systems, which can be useful as thickening and gelling agents (Blennow *et al.*, 2003; Weissenborn *et al.*, 1994). Approximately 18% of the European potato crop is used for starch production, although this varies by country and up to 60% of the potato crop in Denmark is used for starch extraction (Davies, 2002).

Crop production is limited by the availability of water and as climate change accelerates it will impact food security and add to the many economic and social challenges currently faced by world agriculture. The shallow and sparse root system of the potato makes it sensitive to drought stress; therefore, irrigation is important to ensure a high-potato yield and the increasing worldwide shortage of water resources will have an impact on potato production and reduce the tuber yield (Jefferies & Mackerron, 1993; Porter *et al.*, 1999). In comparison to other crops, the potato is the most energy productive crop, however, the availability of water can affect the nutrient availability, nutrient uptake and efficiency and the nutrient composition of the plant itself (Renault and Wallender, 2000). Due to the high productivity per unit area of land, the time taken to grow and the potato's value as both a staple and cash crop, increasing the crop tolerances to stress could help strengthen its contribution to the food and agricultural industry. When compared to other staple crops, a greater proportion of the potato is edible (up to 85% of the plant is edible whilst only 50% is edible in cereals) and a higher yield per hectare is achieved which, combined with its high nutritional value underlines the potato's importance and ongoing increase in global potato production.

1.4 Modern Potato Breeding and Advancements

Modern potato breeding began in 1807 with the first deliberate crosses between varieties, continuing in Europe and North America during 19th century when many new cultivars were produced (Knight, 1807). Modern potato breeding in China and India did not occur until the 1930s, however these countries have now become two of the leading potato producing countries (Srivastava *et al*., 2016). There are now over 4,000 recognised potato cultivars, however it is believed that the genetic base of potato

breeding originates from a relatively small sample of clones from the Andes and coastal Chile, although these must have contained a large amount of genetic diversity (Birch *et al.*, 2012). To meet the increasing demand for food in relation to human population growth, there is a need for increased and stable potato production, and new cultivars must deliver high yields with lower inputs, overcome disease challenges and environmental stresses whilst also possessing improved nutrition properties (Gaur & Pandey, 2000).

Solanum tuberosum and closely related species are typically self-incompatible, making cross-breeding necessary (McKey *et al.*, 2010). Selection of higher yield cultivars has resulted in potato plants producing less flowers and fruits in order to reduce the amount of energy output and concentration on the production of tubers; however, this also reduces the potential for reproduction (Simmonds, 1997). The cultivation of potato relies heavily on clonal propagation, consequently, approximately 10% of the potato tubers produced annually are used for propagation rather than consumption (Spillane *et al.*, 2004). Clonal propagation has a number of benefits and ensures that specific biochemical and physical traits are preserved (McKey *et al.*, 2010). Purely clonal potato crops can, overtime, accumulate mutations, mixing with other varieties and become infected with pathogens that may be present in other plants or in the soil (McKey *et al.*, 2010). Many countries have established seed certification systems to ensure lineage standards and the health of the seed potatoes are maintained, whilst also limiting the number of generations allowed in order to filter out older seed (SASA, 2015).

Sustainable potato production relies on a continual supply of disease-free planting material and seed production has historically been most successful in countries with cooler climates. In cooler climates, there are fewer insect vectors which reduce the problems associated with virus and phytoplasma diseases (Birch *et al.*, 2012). Micro-propagation, or *in vitro* tissue culture, was adopted in many countries in the 1970s as a means to ensure the multiplication of disease-free plants as the process begins under sterile laboratory conditions. Plants are then transferred to clean glasshouses to produce minitubers. Whilst this process is effective, it is expensive and less adoptable in poorer countries (Birch *et al.*, 2012). In 1999, the European and Mediterranean Plant Protection Organisation (EPPO) published an updated certification scheme which includes recommendations for the testing of initial seed potatoes produced by micro-propagation, with the conditions and tolerance for various categories aligned with those that had been set up previously by the UNECE standards (OEPP/EPPO, 1999; UNECE, 2011).

1.5. Potato Genomics

The study of potato genetics has proved challenging, even with the advancements in genetic technologies. *Solanum tuberosum* is a tetraploid displaying tetrasomic inheritance. It has relatively numerous but small chromosomes, which makes genetic studies difficult (Yeh and Peloquin, 1965). Genetic studies have been facilitated by the production of haploids of *S. tuberosum* with other fertile dihaploids; however, it has not been possible to achieve the same degree of sophistication as found with the genetic analysis of crosses between true-breeding inbred lines that display disomic inheritance such as tomato, rice and barley (Hougas *et al*., 1958; Birch *et al.*, 2012). The first molecular marker map was developed in 1988 and greatly increased the genetic understanding of potato and the availability of larger volumes of potato sequence data, initially in the form of expressed sequence tags (ESTs) and sequenced PCR amplicons, has led to the development of single nucleotide polymorphisms for potato (Bonierbale *et al.*,1988*;* Rickert *et al.*, 2003).

The Potato Genome Sequencing Consortium (PGSC) was formed in 2004 to use the genotype RH89-039-16 as the sequenced genotype; however in 2009 the genotype used was switched to a fully homozygous genotype, a 'doubled monoploid' of *Solanum tuberosum* Group Phureja clone DM (DM1-3 516 R44). A 'whole genome shotgun' (WGS) sequencing strategy was employed using a combination of various Next Generation Sequencing technologies, enabling rapid elucidation of the potato genome sequence (The Potato Genome Consortium 2011). The DM genome assembly is of high quality with more than 95% of the genome genetically anchored. The potato genome appears to have undergone extensive genome duplication and, in comparison to tomato, there appear to be nine major chromosomal inversions, more than supposed from previous genetic studies (Tanksley *et al.*, 1992).

Potato breeding involves making crosses between pairs of parents with complementary features to generate genetic variation allowing for phenotypic selection across vegetative generations and for the selection of desirable characteristics (Birch *et al.*, 2012). Advancements in the understanding of potato genomics facilitate the appropriate selection of parents known to possess the desired major genes and quantitative trait locus (QTL). Potato breeding initially involves the selection at an early generation stage and the first and second clonal generations are raised at a high-grade seed site with a short growing season (Bradshaw and McKay, 1994; Bradshaw *et al*., 2003). The whole

process of developing new potatoes can take up to nine years with the intention of producing new cultivars that produce good yields, are agronomically strong, possess few internal defects and express good cooking and processing characteristics, in addition to assessing their resistance to pests and disease (Bradshaw *et al.*, 2003).

1.6 Seed Classification Schemes

The use of Seed Classification Schemes also maintains the genetic purity of the seed and inspections of the seed and fields keep the seed free from pathogens (Albrechtsen, 2006). Since the 1960s, the United Nations Economic Commission for Europe (UNECE) have drafted recommendations for an international standardisation in seed certification, and seed certification schemes provide clear regulations and labelling throughout the whole process of production, harvesting, storage and trade for each seed lot (UNECE, 2011). The current production of agricultural seed in the EU is controlled by an EU-wide framework, and it is intended that the standards set by UNECE be adopted globally as reference to facilitate fair international trade. An international seed certification system would define the quality requirements for seed potatoes to facilitate, support and maintain pedigree records and the quality of distributed seed and other propagation materials (UNECE, 2011). In combination with these standards, the International Plant Protection Convention (IPPC) provides phytosanitary certificates to help prevent the global spread and introduction of pests and pathogens as part of the FAO Plant Protection Service (Birch *et al.*, 2012).

1.7 Scottish Potato Production

Scotland is a major producer of quality seed potatoes and seed produced in Scotland is classified under the Seed Potato Classification Scheme (SPCS). SASA is the Certifying Authority for seed potatoes in Scotland. The Seed Potatoes (Scotland) Regulations 2000 and Seed Potatoes (Scotland) Amendment Regulations 2005 and 2007 requires that seed stocks derived in Scotland must originate from nuclear stock produced by SASA which ensures that the starting material is pathogen-free. This implements the requirements of EC Council Directives 93/17/EEC and Land Commission Decision 2004/3/EC. The nuclear stock is maintained by SASA on behalf of the potato breeders and the industry, and is regularly tested for indigenous and EU-quarantine pathogens. SASA maintains a collection of over 1000 varieties which can be issued to approved micro-propagation laboratories for maintenance and further multiplication. Micro-plants are grown in a

pest free medium in a protected environment to produce minitubers and the classification of the seed tubers are described below (SASA, 2015).

Scotland is recognised as a Community Grade region within the EU, thereby, stricter health standards are applied to Scotland than elsewhere in the EU and consequently the country produces and markets only pre-basic and basic seed potatoes (SASA, 2015). Only seed potatoes that are classified at Community Grade level can be introduced into Scotland from the rest of the EU which safeguards against the introduction of such pathogens as *Clavibacter michiganensis* ssp. *sepedonicus* (ring rot), *Ralstonia solanacearum* (brown rot) and *Potato spindle tuber viroid*, which have never been found in potatoes in Scotland. Imports of seed potatoes from outside of the EU are permitted only through quarantine units (SASA, 2015). Regulations also require that seed crops in Scotland are only grown on land that has been free of potato cultivation for the preceding five years for basic classification and seven years for pre-basic classification. In addition, the land must be free from potato cyst nematodes (*Globodera rostochiensis* and *Globodera pallida*) and must never have had an occurrence of wart disease (*Synchytrium endobioticum*).

Initial (nuclear) stock, from which all Scottish seed crops are derived, is produced by micro-propagation in the government laboratories at SASA. These tissue cultures are subject to stringent testing to ensure freedom from pathogenic organisms. Further multiplication is carried out by officially approved commercial micro-propagation facilities to produce disease-free minitubers (pre-basic TC). These minitubers are then released to officially approved growers for cultivation in the field as pre-basic. There is an official limit to the number of generations that can be produced at each class ensuring older stocks are flushed out. The health standards at each category must be assured by at least two official crop inspections per growing season and any crops that do not meet these standards are rejected from the classification scheme and cannot be marketed as seed. This ensures that the seed from Scotland remains of a high standard, typically higher than most other exporting countries. The following six classes of seed potato are produced in Scotland and overseen at SASA: nuclear stock, pre-basic TC, pre-basic, basic S, basic SE and basic E, and the propagation and disease limitations are described below:

Pre-Basic TC (minitubers)

These stocks are grown in pathogen-free medium in a protected environment by officially approved growers for one generation only. They are produced from selected clones which have been initially propagated from micro-plants in tissue culture, which have been prepared and tested by SASA to be free from viruses in addition to a number of fungi and bacteria, particularly those that commonly cause latent infections.

Pre-Basic

These minitubers are permitted to be planted in the field for up to four generations for classification as pre-basic. All stock must be 99.9% pure and true to type, in addition to being completely free from *Tobacco vein necrosis virus*, *Potato virus Y*, *Potato virus A* and *Leafroll virus*, Blackleg (*Pectobacterium* and *Dickeya* spp.) and Witches' broom phytoplasma at growing crop inspections. These stocks form the basis of seed potato production in Scotland and therefore are not usually marketed outside of Scotland in order to provide a continuous input of healthy material to ensure the high standard of all stocks.

Basic S

These crops are derived from pre-basic seed and must maintain the requirements of 99.9% pure and true to type at growing crop inspection. The disease tolerances are more lenient, with 0.02% of *Potato virus Y, Leafroll virus* and *Potato virus A*, 0.2% for total virus and 0.1% for *Pectobacterium* spp. and zero tolerance for *Dickeya* spp. If these tolerances are met, classification as basic S is allowed up until five generations in the field.

Basic SE

These crops are grown from pre-basic or S stocks and must meet the following tolerances if they are pure and true to type: 0.1% for *Potato virus Y, Leafroll virus* and *Potato virus A,* and a tolerance of 0.5% for *Pectobacterium* spp. and total virus. There remains no tolerance for *Dickeya* spp. If these requirements are met, then the crop can be considered SE up until six field generations.

Basic E

E crops are grown from PB, S or SE crops and if 99.9% pure and true to type, they can be classified as E up until seven generations in field as long as the following disease tolerances are met: 04.% for *Potato virus Y, Leafroll virus* and *Potato virus A* and a total virus tolerance of 0.8%. There remains zero tolerance for *Dickeya* spp. but a tolerance for *Pectobacterium* spp. of 1%.

Official inspections are carried out before Scottish seed potato can be marketed and phytosanitary certificates issued, to check the tubers for pests, diseases, damage and defects. Seed lots are given official labels which confirm the identity and classification of the seed in addition to the crop identification number which allows all Scottish seed to be traceable.

1.8 Threats to Potato Production

Pests and diseases are a continual and serious threat to potato production. Where possible, pesticides have been applied to control disease and chemical control has been increasing in the developing world as the production of potato has increased. A significant increase in potato production would be achieved if only a quarter of the diseases found on potatoes could be controlled in this way; however, there has been considerable political pressure to reduce chemical control of disease, due to the risks these chemicals pose to health and the environment (Gebhardt and Valkonen, 2001). In Europe, the conditions under which chemicals are approved for agriculture is controlled by the EU Directive 91/414/EEC and limits the availability of effective fungicides and bans the use of many effective nematicides. Many of the companies which produce these chemical controls are based in Europe, resulting in a knock-on effect and reduction in the availability of the controls outwith Europe (Birch *et al.*, 2012). There is a significant need to develop alternative means for pest/pathogen control and disease resistance.

The major global diseases of potatoes are late blight, nematodes, the bacterial threats of brown rot, ring rot and blackleg (caused by *Pectobacterium* and *Dickeya* spp.) and viruses.

Late blight

The most widespread and economically significant threat to potato production is the disease late blight, which is caused by the oomycete pathogen *Phytophthora infestans* (Haverkort *et al.*, 2009). *Phytophthora infestans* is airborne and infects the foliage, tubers and stems of a variety of solanaceous crops (Birch and Whisson, 2001). The current means to prevent late blight disease is the application of fungicides; however, the sequencing of the *P. infestans* genome in combination with the identification of effector genes indicates that this pathogen adapts readily, and also serves as a tool to develop cultivars resistant to this disease (Haas *et al.*, 2009; Birch *et al.*, 2008; Schornack *et al.*, 2009; Vleeshouwers *et al.*, 2011).

Nematodes

Nematodes can attack a number of plants, including potatoes, through a variety of feeding mechanisms. In some instances, the plant provides a transient food source whilst for other the interactions are more complex and long-lasting. Nematodes including *Trichodorus* and *Paratrichodorus* are the most important migratory ectoparasites in potato production and cause stunted or deformed roots whilst also being capable of transmitting a number of plant viruses such as *Tobacco rattle virus* (TRV) (Riga and Neilson, 2005; Ploeg *et al.*, 1992). The most important nematodes in potato production are the potato cyst nematodes (PCN), *Globodera rostochiensis* and *Globodera pallida*, which have highly complex interactions with their hosts (Birch *et al.*, 2012). PCN have a restricted host range and juveniles only hatch when the suitable hosts are detected by molecules released from the roots, which the juveniles then invade and migrate to the inner cortex of the plant through the root tissue and induce the formation of large, multinucleate syncytium. Damage caused by PCN is related to the level of infestation and if left uncontrolled are capable of causing up to a 75% loss in potato yield (Seinhorst, 1982). Control of PCN previously relied on a combination of natural resistance combined with nematocides. Many of the nematocides have been removed under the EU directive (91/414/EEC) which limits the ability of growers to control PCN (Birch *et al.*, 2012). Genome sequencing of *G. pallida* will facilitate the identification of new, specific control targets, such as enzyme pathways, and can help guide breeding of resistance against PCN, such as the identification of virulence genes (Sacco *et al.*, 2009; Jones *et al.*, 2009).*Ralstonia solanacearum*

Ralstonia solanacearum can cause disease in over 200 plant species, the most economically important of which being potato, tobacco, tomato and banana (Allen *et al.*, 2005). It can spread through contaminated soil and debris and also through weed hosts and irrigation water; latently infected seed potato accounts for the spread of *R. solanacearum* over longer distances (Van Elsas *et al.*, 2000; Granada and Sequeira, 1983; Graham *et al.*¸1979). *Ralstonia solanacearum* causes bacterial wilt on potato and affects around 3 million growers on 1.5 million hectares of land in over 80 countries, with yield losses in some regions as high as 90% in the field and 98% storage and estimated losses of over $950 million annually (Walker and Collion, 1998). In many countries it is considered to be one of the top five most damaging pathogens of potato. Temperature plays an important role in the development of disease by *R. solanacearum*, with optimal temperatures being 24-35ºC (Swanepoel, 1990). Lower temperatures often result in latent infection which facilitates the spread of disease over further distances (Nyangeri *et al.*¸1984). No chemical controls are available for bacterial wilt and soil fumigants and the use of antibiotics have proved ineffective (Murakoshi and Takahashi, 1984; Czajkowski *et al*., 2013). Limited success has been achieved using biocontrol in the form of antagonistic bacteria such as *Pseudomonas brassicacearum* (Zhou *et al.*, 2012). The use of pathogen-free seed, non-cutting of seed potatoes and 5-7 year crop rotations with non-susceptible crops have been recommended as means of reducing the incidence of disease (Berrios and Rubirigi, 1993; Lemaga *et al.*, 2001).

Viruses

Approximately 40 viruses naturally infect potato, but only one-third of them cause economically important diseases (Jeffries, 1998). Of these, the viruses *Potato leafroll virus* (PLRV), *Potato virus X* (PVX), *Potato virus Y* (PVY), *Potato virus A* (PVA), *Potato virus S* (PVS) and *Potato virus M* (PVM) commonly occur in potato production systems worldwide (Valkonen, 2007). Symptoms associated with virus infections include curling, yellowing or mosaic symptoms on leaves, stunting of plants, and some affect tuber quality, inducing brown or necrotic marks and lines on tubers, and rarely cause complete destruction of the crop (Valkonen, 2007). The impact of viruses come from their transmission to progeny plants which give rise to small, deformed tubers and can accumulate over several growing seasons which results in significant reductions in yield. Yield losses from PVY-infected seed were 29-59%, however, plants can be infected by more than one virus simultaneously which can lead to more severe symptoms through synergism, which also makes it difficult to diagnose the infecting

virus (Valkonen, 2007). Similar to other pathogens, temperature can influence the expression of symptoms with cooler temperatures (12-16^{o}C) favouring symptoms induced by PVS, PVM, and *Potato mop top virus* (PMTV) and warmer temperatures (18-20^{o}C) favouring PVY (Peters, 1987).

Transmission of the most economically important potato viruses are through insects, primarily aphids, however some, such as *Tobacco rattle virus* (TRV), are soil-borne, transmitted by nematodes or can be spread from weed reservoirs through water to the tubers (van Hoof, 1968; Jones & Harrison, 1969; Birch *et al.*, 2012). The most effective method in controlling viruses is through host resistance and a number of *Solanum* spp. have been identified which are resistant to particular viruses (Solomon-Blackburn and Barker, 2001).

Dickeya and *Pectobacterium sp.*

Ralstonia solanacearum may be the major bacterial pathogen of potato in developing nations; but, the impact of *Pectobacterium* and *Dickeya* spp. has long been known in South America and is increasingly being recognised as having an impact on potato production in countries on other continents, with different species favouring different climates (Duarte *et al.*, 2004; Pérombelon, 2002) The pathogens belong to the bacterial family *Enterobacteriaceae*, which cause a variety of soft rot diseases on a number of plant species, including tomato, banana and maize; although, it has the most economic impact on potato (Pérombelon, 2002). As seen in *R. solanacearum*, both *Pectobacterium* and *Dickeya* spp. are predominantly seed-borne and distributed over long distances through contaminated seed (Pérombelon, 2002).

1.9 History of *Dickeya* spp.

The genus *Erwinia* was established in the 1920s to encompass all members of the *Enterobacteriaceae* that were pathogenic to plants including both pectinolytic and non-pectinolytic, with *Erwinia chrysanthemi* being assigned to the genus in 1953 by Burkholder *et al.* as a pathogen of chrysanthemum. Later studies revealed that *E. chrysanthemi* caused disease on a wide variety of hosts (Samson *et al.*, 2005; Ma *et al.*, 2007). Because of the wide host range of *E. chrysanthemi* Lelliott and Dickey (1984) subdivided the species into six pathovars based on host specificity. Samson *et al.* (1987) developed a biovar system based on some key biochemical characteristics and in 1998 the name *Pectobacterium* was applied to a number of potato pathogens based on 16S

rDNA analysis (Hauben *et al.*, 1998). *Pectobacterium* had been proposed in 1945 to describe the pectinolytic erwiniae (Waldee *et al.*, 1945). The potato pathogenic *Pectobacterium* included *P. carotovorum* subsp. *carotovorum*, *P. atrosepticum* and *P. chrysanthemi*; however further analysis of *P. chrysanthemi* using 16S rDNA, DNA-DNA hybridisation and biochemical characterisation showed that it forms a distinct clade, given the genus name *Dickeya* (Samson *et al.*, 2005). *Dickeya* was originally divided into six species, listed in Table 1.

Table 1.1 Description of *Dickeya* sp. and the Previous Identifications of the Species (as Adapted from Samson *et al.*, 2005).

Dickeya species	Synonyms (including biovars and pathovars)
D. dianthicola	*Erwinia chrysanthemi* biovars 1,7 &9 *E. chrysanthemi* pv. *dianthicola* *Pectobacterium chrysanthemi* pv. *dianthicola*
D. dadantii	*E. chrysanthemi* biovar 3 *P. chrysanthemi* biovar 3
D. zeae	*E. chrysanthemi* biovar 8 and other strains of biovar 3 *P. chrysanthemi* biovar 8 and other strains of biovar 3
D. chrysanthemi pv. *chrysanthemi*	*E. chrysanthemi* biovar 5 *E. chrysanthemi* pv. *chrysanthemi* *P. chrysanthemi* pv. *chrysanthemi*
D. chrysanthemi pv. *parthenii*	*E. chrysanthemi* biovar 6 *E. chrysanthemi* pv. p*arthenii* *P. chrysanthemi* pv. p*arthenii*
D. paradisiaca	*E. chrysanthemi* biovar 4 *E. chrysanthemi* pv. *paradisiaca* *E. paradisiaca* *Brenneria paradisiaca*
D. dieffenbachiae	*E. chrysanthemi* biovar 2 *E. chrysanthemi* pv. *dieffenbachiae* *P. chrysanthemi* pv. *dieffenbachiae*

Subsequent revision of this genus by Brady *et al*. (2012) reclassified *D. dieffenbachiae* as a subspecies of *D. dadantii*. In 2014, *Dickeya solani* was accepted as a novel species within the genus *Dickeya* encompassing isolates forming a distinct clade based on multilocus sequence analysis (MLSA) using the concatenated sequences of the intergenic spacer (IGS), in addition to *dnaX*, *recA*, *dnaN*, *fusA*, *gapA*, *purA*, *rplB*, *rpoS*, and *gyrA*. Characterisation by whole-cell MALDI-TOF mass spectrometry, pulsed field

gel electrophoresis after digestion of whole-genome DNA with rare-cutting restriction enzymes, average nucleotide identity analysis and DNA-DNA hybridisation studies which showed that, although related to *Dickeya dadantii*, these isolates represent a new species, given the species name *D. solani*, with the type strain being IPO2222 (van der Wolf *et al.*, 2014).

1.10 *Dickeya* spp. on Potato

There is evidence that all previously described *Dickeya* spp., with the exception of *D. paradisiaca*, have been detected on a number of ornamental plants in Europe (Janse & Ruissen, 1988; Samson *et al.*, 2005; Parkinson *et al.*¸2009; Sławiak *et al.*¸2009). *Dickeya dianthicola* and *D. solani* appear to be the only species that have been found on potato in Europe, with the first European report of *Dickeya* spp. on potato in the Netherlands in the 1970s. It has subsequently been found in a number of other European countries and by the early 1990s, *D. dianthicola* had been reported as the most frequently isolated pathogen from the genus on seed potatoes (Cazelles & Schwarzel, 1992; Sławiak *et al.*, 2009; Toth *et al.*, 2011).

Prior to 2004, almost all *Dickeya i*solates from potato were *D. dianthicola*, which was first detected as causing stunting and slow wilting in *Dianthus* in the early 1950s in Denmark, the Netherlands and the UK (Hellmers, 1958; Parkinson *et al.*, 2009). *Dickeya dianthicola* was subsequently listed as a quarantine organism (EPPO A-2) on *Dianthus* (Council Directive 2000/29/EC). Recent studies isolating *D. dianthicola* from eight host plants from Europe showed little sequence variance despite encompassing multiple isolates recovered from *Dianthus* and potato (Parkinson *et al.*, 2009; Sławiak *et al.*, 2009). This suggests that *D. dianthicola* may have successfully spread from other host plants to potato. Since 2007, the majority of blackleg cases caused by *Dickeya* spp. have been attributed to *D. solani* (Toth *et al.*, 2011).

1.11 Economic losses due to *Dickeya* spp.

EPPO currently lists *Erwinia chrysanthemi*, which equates as *Dickeya chrysanthemi* pv. *chrysanthemi* and *D. dianthicola*, as an A2 quarantine organism of carnations and chrysanthemums (OEPP/EPPO, 1982, 1988, 1990). There are no current quarantine implications in Europe for *D. dianthicola* or any other *Dickeya* sp. on potatoes where it has widely been considered that the risk from *Dickeya* spp. can be adequately covered by national nuclear stock and seed certification schemes (Toth *et al*, 2011). In Israel,

however, *Dickeya* spp. are considered to be a quarantine organism and in Scotland, a zero tolerance for *Dickeya* spp. on potatoes was introduced in 2010 as part of the Seed Potato Classification Scheme (Tsror *et al.*, 2009; The Seed Potatoes (Scotland) Amendment Regulations 2010).

In Israel, yield reductions of 20-25% resulting from *Dickeya* infections have been recorded on various potato cultivars where disease incidence was greater than 15% (Tsror *et al.*, 2009). Most direct losses in Europe caused by *Dickeya* have occurred due to the downgrading or rejection of potatoes during seed tuber certification. Since national certification tolerances differ, the economic impact varies by country. In the Netherlands, strict tolerances have led to increased direct losses of up to €30 Million annually due to downgrading and rejection of seed tuber stocks caused by blackleg, as it has not been possible to distinguish between *Pectobacterium* and *Dickeya* spp. (Prins & Breukers, 2008). Crop losses can occur for seed growers, suppliers and exporters if tubers containing *Dickeya* spp. are exported to warmer climates and result in decomposition of the tuber (Toth *et al.*, 2011). With the emergence and spread of *D. solani*, together with the effects of climate change, more frequent losses of this kind can be expected. Data from Israel indicates the scale of potential losses that can be expected when seed tubers latently infected with *D. dianthicola* or *D. solani* are grown in warmer climates, as disease incidences ranging from 5% - 30% was seen on five different potato cultivars (Tsror *et al.*, 2009).

1.12 Factors influencing *Dickeya* Disease Development

Factors influencing disease development by *Dickeya* spp. are similar to those of *Pectobacterium* spp. and include damage and lack of cleanliness at grading, poor soil drainage, presence and increasing level of the pathogen on seed tubers, over-irrigation, wet spring weather, damage at harvest and lack of adequate ventilation at storage (Toth *et al.*, 2011). Factors that influence disease by *Dickeya* spp. over that of *Pectobacterium* spp. are inoculum level, as lower inoculum levels of *Dickeya* spp. are required to cause disease, cultivar susceptibility, although at present, there is limited evidence of the susceptibility of potato cultivars to *Dickeya* spp., the speed of migration through the plants' vascular system, temperature and relative aggressiveness (van der Wolf *et al.*, 2007; Toth *et al.*, 2011). There is circumstantial evidence to suggest that *Dickeya* spp. are better at invading the vascular tissue of potato plants than *P. atrosepticum* and may be found at higher concentrations at the stolon ends of the tubers. They are also thought

capable of infecting roots within one day of soil inoculation (Czajkowski *et al.*, 2009; 2010b).

Temperature is considered the most important factor in determining whether disease in any one season will be predominantly caused by *Dickeya* spp. (Lumb *et al.*, 1986). In Israel, it was found that symptoms caused by *Dickeya* spp. tended to develop when temperatures exceeded 25ºC whilst *P. atrosepticum* predominated below 25ºC (Pérombelon & Hyman, 1986). The relative aggressiveness of the pathogens is also dictated by temperature, with *D. solani* being shown to grow at temperatures as high as 39ºC and expressing more aggressive symptoms than *D. dianthicola* at higher temperatures; *in vitro* studies have also shown *D. solani* to express more variability in aggressiveness (Laurila *et al.*, 2008; Tsror *et al.*, 2009). The opposite may be true at lower temperatures, with studies in Finland showing that *D. dianthicola* resulted in the highest incidence of diseased plants (Laurila *et al.*, 2008).

1.13 Transmission and Survival of *Dickeya* spp.

The most important means of dissemination for bacterial pathogens of potato is the movement of latently infected seed tubers and *Dickeya* spp. are spread over long distances by infected propagating material (Pérombelon & Kelman 1980; Tsror *et al.*, 1999, 2009). The pathogen can be carried on the tuber surfaces and in lenticels but is also likely to be found in the tuber's vascular system which it enters systemically *via* the stolon from the infected mother plant or *via* root infection (Czajkowski *et al.*, 2009, 2010). Other studies have investigated the potential for survival of *Dickeya* spp in water and also in the soil. Studies in the Netherlands found that surface water can be contaminated with *Pectobacterium* spp. and *Dickeya* spp. particularly in the autumn season, although, there is little or no correlation between *Dickeya* spp. isolated from river water and those from potato in Europe (Laurila *et al.*, 2008, 2010). *Dickeya solani* has been isolated from river water in Scotland but not from Scottish seed tubers, and the population densities found in the river water remained constant after repeated annual sampling, eventually disappearing (Cahill *et al.*, 2010). It appears unlikely that *Dickeya* spp. can survive in the soil although it is possible for the pathogen to persist in the presence of crop residues (Velvis & van der Wolf, 2008; van der Wolf *et al.*, 2009). Reports from the Netherlands have shown that potato crops multiplied twice in the field, grown from pathogen-free seed, were observed to have 20-56% infection of *Dickeya* spp. in harvested tubers, implying that the pathogen is either transferred *via*

contaminated farm machinery, can survive in the potato growing environment or is transmitted from outside of the cropping environment (Toth *et al.*, 2011).

Prior to the reclassification of *Erwinia chrysanthemi* into species within the genus *Dickeya* it was unclear whether symptoms caused by *Dickeya* spp. on potato in Europe were similar to those of *Pectobacterium atrosepticum* or were sufficiently different to allow disease diagnosis (Toth *et al.*, 2011). There is likely to be no simple correlation between symptomology between *P. atrosepticum* and *Dickeya* spp. but rather that there may be a range of symptoms depending on the species, isolate, environmental conditions and even the cultivar used. Generally it appears that *Dickeya* spp. causes stem rotting with symptoms similar to those of *P. atrosepticum* under warm, wet conditions and when the humidity is lower, less rotting is observed but wilting, leaf desiccation, stem browning and hollowing of the stem is more likely (Palacio-Bielsa *et al.*, 2006; Tsror *et al.*, 2009).

1.14 The importance of Diagnostics of *Dickeya* spp.

Contaminated seed tubers play an important part in the introduction of *Dickeya* spp., in addition to the use of contaminated machinery and equipment during cultivation, harvesting and grading and possible environmental contamination. Biosecurity measures should be employed in areas where *Dickeya* spp. are not present to prevent the introduction of the pathogen. Infected seed tubers represent the most likely source of introduction to a new area, with almost all new findings of *Dickeya* spp. being traced back to the seed source (Lumb *et al.*, 1986; Sławiak *et al.*, 2009; Tsror *et al.*, 2009). Diagnostic tests can effectively be used to identify the presence of *Dickeya* spp. which can help to avoid planting or exporting infected stocks. Previously, differentiation between *Pectobacterium* and *Dickeya* spp. was purely through visual inspection which can miss latent infections. Diagnostic tests are intermittently applied in most countries and are usually voluntarily (Toth *et al.*, 2011). The most commonly used method for the detection of *Dickeya* spp is isolation on selective crystal violet pectate medium (CVPM) with a double-layer modification recently used to isolate species of *Pectobacterium* and *Dickeya* (Cuppels & Kelman, 1974; Hélias *et al.*, 2011). Incubation at different temperatures for 48 hours: 36^{o}C for *Dickeya* spp. isolation and 25^{o}C for *P. atrosepticum*, can be used to isolate species. Serological tests have also been used to screen seed potatoes for latent infections but have generally been found to be lacking in specificity and sensitivity for *Dickeya* spp. (Samson *et al.*¸1990; van der Wolf *et al.*,

1993). For routine screening, PCR-based assays are increasingly being used and the most widely used PCR assay for the detection of *Dickeya* spp. is based on the pectate lyase gene *pel* using the primers ADE1/ADE2 designed by Nassar *et al.* (1996). Alternative PCR assays have also been developed although these identify the soft rot *erwiniae* as a whole, rather than individual *Dickeya* species (Toth *et al*. 1999; 2001). A multiplex PCR assay has also been designed for detection of both *Dickeya* and *Pectobacterium* spp. and relies on the primer pairs Y45/46, which targets *Pectobacterium atrosepticum* and ECH1/1' from the *pel* gene which targets *Dickeya* sp. (Fréchon *et al.*, 1998; Diallo *et al.*, 2009). The most effective way to identify *D. solani* has been through selection using growth on CVPM, followed by screening using the Nassar assay for *Dickeya* spp. and identifying the species through sequencing of either the *recA* or *dnaX* gene (Parkinson *et al.*, 2009; Sławiak *et al.*, 2009).

1.15 Aims and Objectives of PhD project

The emergence of *Dickeya solani*, which is responsible for significant potato crop losses, is a major concern as it can cause disease in both cool, wet conditions and warmer dryer conditions. This may lead to the increased prevalence of blackleg and related diseases over a wider range of weather conditions. The increased spring and summer temperatures, arising as a result of climate change, may exacerbated the problem as *Dickeya* spp, especially *D. solani*, are more aggressive at higher temperatures. Increased trade is also playing a major role in the spread of disease and the distribution of infected seed tubers is believed to be the main cause of the spread. Once in a potato crop, the effects of *Dickeya* spp. can be serious and are not easily differentiated from *Pectobacterium* spp. based on symptomatology. It is therefore important that diagnostic tools be developed to allow differentiation between *Dickeya* spp.. These will be useful in future epidemiology studies. Diagnostic tests will also be essential if new legislation to prevent the spread of *Dickeya* spp., such as the zero tolerance policy in Scotland, is to be effective. Other control measures are largely based on those for *Pectobacterium* spp. but if *Dickeya* disease is to be controlled, research is needed to identify specific measures for *Dickeya* spp., therefore the aims of this project are outlined below:

The objective of this project was to understand the biology and epidemiology of *Dickeya solani*, a recently emerged bacterial pathogen of potato. Within this objective, the research has a number of more specific aims:

1. To refine, validate and apply diagnostic, detection and typing methods specifically for *Dickeya solani* to assist with designing adequate control measures and understand the introduction and evolution of the pathogen.

2. To evaluate the risk of spread to Scottish grown seed potatoes by understanding the transmission of the pathogen from infected tubers under field conditions.

3. To improve the understanding of the transmission and survival of *Dickeya solani* in storage and the susceptibility of *D. solani* to common disinfectants to improve control of the pathogen once introduced to a crop or seed lot.

Chapter 2. The Development of a Real-Time PCR Diagnostic Assay specific for *Dickeya solani*

2.1. Introduction

2.1.1 *Potato Industry of Great Britain*

The rise in the global trade of plant material has facilitated the spread of plant pathogens, therefore, it has become even more important that seed is free from infection and that there are strict and efficient monitoring and policy measures in place to limit the spread of disease (Toth *et al.*, 1999). The European and Mediterranean Plant Protection Organisation (EPPO) currently lists 26 phytopathogenic quarantine organisms which cause significant yield losses in cultivated plants, decreased agriculture production and threaten natural ecosystems. Accurate detection of pathogens is crucial for virtually all aspects of plant pathology. The implementation of management and control strategies are necessary to prevent the introduction of pathogens and require the rapid and reliable identification of pathogens without unduly obstructing trade (Fletcher *et al.*, 2006; Pelludat *et al.*, 2009). Prevention of the dissemination of pathogens is more effective than control of the pathogen within production, and prevention relies on highly sensitive, specific and reliable detection methods, to ensure the identification of pathogens and to prevent their incorporation into the production chain, especially as many bacteria either remain latent or are present in low numbers within plant material (López *et al.*, 2009).

2.1.2 *Diagnostics and Detection of Potato Pathogens*

Plant health status is dependent on diagnostics and detection, with diagnostic tests identifying pathogens present in material expressing symptoms and detection focusing on symptomless plant material (López *et al.*, 2009). Decisions relating to plant protection and the implement of quarantine control measures require rapid and reliable identification (Pelludat *et al.*, 2009). Classical detection methods, such as isolation, are typically sensitive; however, with the limited population of organisms in plant material such as seeds, the sensitivity of such tests is reduced; fortunately, considerable progress has been made in the detection of low numbers, even when other organisms are present (Schaad *et al.*, 2007). Detection of plant pathogens previously relied on methods including isolation on specific growth media, serology and bioassays; but there are drawbacks to these methods such as their limitation in targeting a single pathogen, the

requirement of viable cells for isolation and the possibility of limited population numbers (Pelludat *et al.*, 2009). The EPPO standard protocols attempt to overcome these drawbacks by incorporating a combination of techniques to ensure isolation, identification and verification and suggest that when more than one method based on different biological principles are available, that all available tests are used to ensure maximum accuracy. These standard protocols, incorporating isolation and further identification, are time-consuming and not guaranteed to be sensitive or specific enough and are not suited for routine analysis of a large number of samples (López *et al.*, 2009).

2.1.3 *Molecular Advancements in Techniques for Diagnostics and Detection*

The development of molecular (nucleic acid) diagnostic methods has significantly improved the detection of plant pathogens and the range of targets reliably diagnosed using molecular methods has increased significantly with very few plant pathogens without laboratory-based diagnostics (Mumford *et al.*, 2006). Molecular techniques have been in use since the 1970s when double-stranded DNA and dot-blot hybridisation protocols were developed for the detection of viruses and viroids. After the development of Polymerase Chain Reaction (PCR) in the 1980s, molecular techniques began to make a significant impact, with the first use of PCR in plant pathology published by Puchta and Sanger in 1989 (Mumford *et al.*, 2006). Subsequent technological advances in PCR-based methods have enabled fast, accurate detection of pathogens; in addition to the ability to quantify and characterise, the use of molecular diagnostics provides the degree of discrimination needed to detect and monitor plant diseases that is not always possible through other types of analysis (López *et al.*, 2009). A compilation reports that more than two hundred PCR protocols for the detection and identification of more than 50 bacterial species, 9 subspecies and more than 40 pathovars is now available (Palacio-Bielsa *et al.*, 2009).

Molecular methods are based on specific genomic sequences (oligonucleotides/probes) and the DNA sequences used to design primers can originate from three main types of genes: pathogenicity/virulence genes, ribosomal genes and plasmid genes. The pathogenicity genes used as targets can be involved in any of the several steps leading to symptom development and can be related to virulence factors, virulence genes, toxin products and other factors, or can be located on the chromosome and be specific to a pathogen or a group of pathogens. The use of pathogenicity genes to design primers has been demonstrated for a wide range of bacterial species; but there is also a need to

design new primers for some bacterial strains that lack some of the previously considered universal pathogenicity genes and different strategies have been proposed to design more appropriate PCR primers (Pritchard *et al.*, 2013).

2.1.4 *Polymerase Chain Reaction*

PCR has become a valuable tool for both basic and applied studies of plant pathology and there are several advantages that such a technique offers over traditional methods (López *et al.*, 2009). The target pathogen does not need to be isolated and cultured prior to detection, the principle of PCR makes it highly sensitive and allows for a single molecular target to be detected within a complex mixture and it is rapid and versatile (López *et al.*, 2009). There are some drawbacks to the use of PCR including the potential for contamination, the sensitivity of the protocol to inhibitors, the cost and the complexity of design as the success of the assay is based on primer specificity in addition to the polymerase type, buffer composition and stability, the purity and concentration of dNTPs, and the cycling parameters (López *et al.*, 2006). These drawbacks can limit in some cases the use of PCR in routine diagnostics and the method can be difficult for high-throughput diagnostics.

The low detection threshold of PCR makes the technology ideal for use in diagnosing low-level latent infections of important pathogens as PCR has shown sensitivity and specificity at population densities of 1 - 10^3 cells.ml^{-1} (van der Wolf *et al.*, 2001). The development of molecular methods has focused on specificity and sensitivity, and, over time has come to include more practical advancements. To obtain results from PCR, endpoint analysis such as agarose gel electrophoresis is required and the results do not provide information regarding the amount of pathogen in the original sample (Mumford *et al.*, 2006; López *et al.*, 2009).

The advancements made through conventional PCR has led to the development of real-time PCR which has numerous advantages over conventional PCR. The technology of real-time PCR removes the need for endpoint analysis and allows for results to be obtained faster than conventional PCR, makes it possible to quantify the original target population, allows for the detection of several variants of a pathogen and even identification of point mutations within a gene (Deepak *et al.*, 2007). The removal of the endpoint analysis, the use of a closed system and fewer reagents also reduces the risk of contamination (Mumford *et al.*, 2006).

Real-time PCR also provides increased sensitivity and some methods have been used to directly detect some pathogens in soil (Mumford *et al.*, 2006). Although real-time PCR employs a fluorescence-based system, the additional cost from this technology is more than offset by the savings in time and labour. Crucially, real-time PCR allows for the quantification of results and allows for the PCR reaction to be monitored whilst it is underway. The most widely used real-time PCR chemistry is TaqMan® (ThermoFisher Scientific) which was developed in the 1990s and utilises an oligonucleotide probe labelled at opposite ends with a reporter and a quencher dye. The TaqMan® probes (approx. 20-30 bases) are longer than the primers and are designed to anneal with 5' exonuclease of Taq polymerase to a sequence internal to the PCR primers. When the probe is intact the fluorescence that is emitted by the reporter is absorbed by the quencher but during amplification, the probe is cleaved by the nuclease activity of Taq which separates the dye and results in an increase in fluorescence which is related to the amount of product amplified (Mumford *et al.*, 2006). A combined thermal cycler and fluorescence reader system monitors the reporter fluorescence during the amplification process, removing the need for post-PCR manipulations such as gel electrophoresis to visualise the results (Mumford *et al.*, 2006).

2.1.5 *Detection Methods for* Dickeya *spp.*

The current diagnostic method for *Dickeya* spp. is time-consuming and laborious. For many years the detection and identification of pectinolytic bacteria was solely through the isolation of viable bacterial cells on semi-selective culture agar media followed by serological and biochemical analysis, bioassays and microscopic observation, however, these methods are cumbersome and time consuming and have been replaced by more advanced molecular methods. Current methods still utilise previous techniques, with the first step in *Dickeya* spp. detection still requiring the isolation of *Dickeya* spp. from samples followed by the growth and purification of the bacteria prior to analysis using molecular methods (Czajkowski *et al.*, 2015). When bacterial populations are low, it is possible to enrich the sample by incubating under anaerobic conditions in Pectate Enrichment Broth (PEB), a liquid enrichment media further described in Appendix A2.7, containing polypectate which mimics conditions that stimulate the natural selection for *Dickeya* and *Pectobacterium* spp. and reduces contamination, allowing the multiplication of the pathogens to facilitate bacterial isolation and subsequent DNA isolation (Pérombelon and van der Wolf, 2002). The most commonly used and preferred isolation media for pectinolytic bacteria is the crystal violet pectate media (CVPM)

which is selective for both *Pectobacterium* and *Dickeya* spp. (Cuppels & Kelman, 1974; Hélias *et al.*, 2011). The selectivity of the CVPM is derived from the presence of crystal violet which inhibits the growth of Gram-positive bacterial and polypectate, which is the source of carbon. *Pectobacterium* spp. and *Dickeya* spp. break down the pectate in the media, creating pits (Cuppels & Kelman, 1974). Growth on CVPM and incubation at temperatures of either 27^{o}C or 36^{o}C allows for the differential selection of *P. atrosepticum*, *P. carotovorum* and *Dickeya* sp., although this method is not always reliable (Pérombelon and Hyman, 1986; Toth *et al.*, 2011). Serological tests have been used to screen seed potatoes for latent populations of *Pectobacterium* and *Dickeya* spp. but they lack the required specificity and sensitivity and false positive results and sensitivity limitations remain a drawback to the use of serological methods for *Dickeya* detection (van der Wolf *et al.*, 1993; Toth *et al.*, 2011).

Molecular detection methods based on the analysis of bacteria genomic DNA have become the most frequently used methods for detection and differentiation of blackleg and soft rot pathogens and consist of the amplification of target-specific sequences (Laurila *et al.*, 2010). The development of PCR assays have advanced the routine screening for *Dickeya* sp., with the most common using the ADE1/ADE2 primers designed from the pectate lyase (*pel*) gene (Nassar *et al.*, 1996). Other PCR methods for *Pectobacterium atrosepticum* and *Dickeya* spp. as a group also exist (Toth *et al.*, 1999, 2001; Smid *et al.*, 1995; van der Wolf *et al.*, 1995) and a multiplex PCR that applies the Y45/46 primers (Fréchon *et al.*, 1998) which targets *P. atrosepticum*, and ECH1/1' derived from the *pel*I gene, which targets *Dickeya* spp. has also been developed (Diallo *et al*., 2009). The most effective way to identify *D. solani* currently is the use of the Nassar PCR assay to screen for *Dickeya* spp., differentiating between species by sequencing either the *recA* gene (Parkinson *et al.*, 2009) or *dnaX* gene (Sławiak *et al.*, 2009); however, this entire process is laborious and expensive (Toth *et al.*, 2011).

2.1.6 *Specific Aims of Developing PCR Assay*

Real-time PCR assays for the detection of soft rot *Enterobacteriaecae* have been developed to enhance the specificity, reliability and quantification of the pathogens. Prior to thi study, only two real-time PCR assay had been developed for the detection of these pathogens and utilise the SYBR green chemistry which is less specific than TaqMan® as it does not use an additional probe to the primer pair (ThermoFisher Scientific). SYBR Green (Molecular Probes Inc., Life Technologies Corporation) binds

to double-stranded DNA and the resulting DNA-dye-complex absorbs blue light and emits green light (Zipper *et al*., 2004). Real-time PCR assays have a great potential for use in plant pathology as it combines identification with quantification of target pathogens

One aim of this study was to design a real-time PCR assay that is specific to *Dickeya solani* with the intention of providing a diagnostic test that is highly specific and reduces the time taken detect *D. solani* from samples. It incorporates the work of Pritchard *et al.* (2013) which designed primers based on raw genome data from various *Dickeya* strains in combination with a bio-informatics tool to predict primer sets.

2.2 Materials and Methods

2.2.1 *Cultures*

A total of 73 *Dickeya* and *Pectobacterium* isolates were assembled, the details given in Table A.1 in Appendix 1. Cultures were stored and maintained as described in Appendix 2, at -80^{o}C in cryovials. Prior to use, two beads were spread onto CVPM and incubated at 36^{o}C for *Dickeya* sp. and 25^{o}C for *Pectobacterium atrosepticum* for 48 hours to ensure purity based on the presence of pit-forming colonies. Colonies were re-isolated onto Nutrient Agar for 24 hours at the appropriate temperatures as previously indicated.

2.2.2 *Real-Time Assay*

Two assays, described in Table 2.1, were evaluated in this study; one designed jointly by JHI, Dundee and Fera, York and referred to as SOL-C, and the other from a previous MLSA study at SASA (referred to as *fusA*) (Kowalewska *et al.*, 2010). The two real-time PCR assays were both designed using comparative genomics. SOL-C was designed at JHI using a bioinformatics pipeline utilising the sequence data of four *D. solani* strains alongside 21 other *Dickeya* spp. strains. Using this method, 1000 PCR primer pairs were identified, the specificity of which were analysed computationally (Pritchard *et al.*, 2013). Following this analysis, a list of potential primer pairs and probes specific only for *D. solani* was collected and were further analysed in the laboratory at Fera against the reference strains of *Dickeya* spp.. The single assay, SOL-C, was selected for the specific identification of *D. solani*.

The *fusA* assay was designed by mining genes encompassed within a previously designed multilocus sequence analysis (MLSA) system. This system had been designed to discriminate *Dickeya dianthicola* strains from related species, including *D. solani* (Kowalewska *et al*., 2010). Initial gene targets were selected through visual assessment of each phylogenetic tree from the MLSA systems, selecting genes showing good separation of the *D. solani* strains. Using this method, *fusA* was selected as the gene target and primer pairs and probes designed by aligning the *fusA* sequences of 62 *Dickeya* and *Pectobacterium* strains using the ClustalW methods and the MegAlign programme in Lasergene V.7.0.0 (DNASTAR inc).

Table 2.1. Primers and Probes Designed for Specific Detection of *D. solani* by JHI/Fera and SASA.

SOL-C	Forward primer (5'-3')	GCCTACACCATCAGGGCTAT
	Reverse primer (5'-3')	ACACTACAGCGCGCATAAAC
	Probe* (5'-3')	CCAGGCCGTGCTCGAAATCC
FusA	Forward primer: DsolfusA-229F (5'-3')	GGTGTCGTTGACCTGGTGAAA
	Reverse primer: DsolfusA-300R (5'-3')	ATAGGTGAAGGTCACACCCTCATC
	Probe*: DsolfusA-250T (5'-3')	TGAAAGCCATCAACTGGAATGATTC

*Probe is labelled with 6-FAM

2.2.3 *Validation of Real-Time Assays*

The SOL-C and *fusA* assays were tested against the collection of a total of 73 *Dickeya* and *Pectobacterium* strains held at SASA. These included strains from the initial MLSA system (Kowalewska *et al.*, 2010) and additional strains later collected by SASA. Emphasis was placed on the *D. solani* isolates to confirm the specificity of these tests for this organism. The strains had been stored on cryovials and were revived as described in Appendix 2. DNA was extracted according to the method described in Appendix 2.

Reaction mixtures were as described in Table 2.2 using the primer/probe pairs indicated in Table 2.2. Master mix (24µl – without DNA sample) was added to wells of a 96-well plate (MicroAmp optical well plate with barcode) using an electronic pipette (Autorep) in a laminar flow cabinet. Either 1µl of boiled cells or extracted DNA was added to each well with 1µl of sterile water used as the negative control. The PCR was completed on an Applied Biosystems 7900HT real-time PCR machine using the conditions described in Table 2.3. The machine was run in standard mode, detecting FAM/TAMRA and using ROX as the passive reference.

Table 2.2. Real-time PCR Reaction Mix for Detection of *Dickeya solani*

Component	Volume per reaction (µl)
Taqman® Fast Universal PCR Master Mix (2X) (Applied Biosystems)	12.5
Forward primer (5 pmol)	1.5
Reverse primer (5 pmol)	1.5
Probe (5 pmol)	0.5
Template	1
Sterile H_2O (Sigma)	8
Final volume	**25**

Table 2.3. Real-time PCR Cycle for Detection of *Dickeya solani*

95°C	10 min	x 1 cycle
95°C	15 s	x 40 cycles
60°C*	1 min	

Data was taken at the extension (*) step only.

A second evaluation was carried out under the same conditions using the core test set for the *Dickeya* EUPHRESCO project, the European collection of *Dickeya* strains (http://www.euphresco.net). The details of the 36 strains from this study are found in Table A.2 of the Appendix.

2.2.4 *Further validation of PCR assays: "Ring Test."*

In order to confirm the results obtained at SASA and support the conclusion that both assays were specific for *Dickeya solani*, an informal ring test was established involving the following laboratories:

1. AFBI, Belfast
2. Fera, York
3. JHI, Dundee
4. NIAB, Cambridge

5. SAC, Aberdeen

6. Potato Council, Sutton Bridge.

At the end of August 2011, in order to begin the validation of the two real-time assays for *D. solani*, ten samples and details of the assays were sent to the participating laboratories, each of which had their own real-time PCR machine. The samples were only labelled numerically and included a mix of spiked sap samples at varying inoculum levels of *D. solani* and related species, and control (un-contaminated sap samples. The details of the strains sent can be found in Table 2.4. Appropriate dilutions of primers and probe reagents (for the assays SOL-C, *fusA* and also ECH (Table 2.5) which positively identifies *Dickeya* sp.) were also included. To test the robustness of the PCR assays, rather than standardising common reagents, participants were advised to use their own DNA extraction protocols. In addition, *Taq* polymerase enzyme and other buffers that were commonly used in each individual laboratory were allowed for the purposes of this study. The protocol for the DNA extraction used at SASA, outlined in Appendix 2 was sent to each participating laboratory for reference. As it is often necessary to enrich environmental samples to increase the number of colonies present, the recipe for Pectate Enrichment Media (PEM) was included in the test and laboratories enriched the samples in addition to testing unenriched samples (Meneley & Stanghellini, 1976; Toth *et al.*, 1999).

In preparation for the ring test, overnight cultures grown on Nutrient Agar were suspended in Extraction Buffer to a concentration of 10^8 c.f.u..ml^{-1}. Potato sap was made from crushing tubers of the cultivar Maris Peer, which had been previously peeled and surface-sterilised using 10% (aq.,v/v) sodium hypochlorite, by placing in a Bioreba bag and using a hammer to disintegrate the tissue. Approximately 20ml of crushed potatoes was added to ¼-strength Ringer's solution (Appendix 2) to bring to a total volume of 50ml. The solution was left at room temperature for 20 minutes then centrifuged at 4,000rpm for 10 minutes at 4ºC. The supernatant was retained and sieved through muslin to remove as much starch and debris as possible. The colony suspension was diluted into the sap solution to give concentrations of either 10^7 or 10^3 cells.ml^{-1}.

Table 2.4. Sap Samples Used for "Ring Test" of *Dickeya solani* Assays

Sample Number	Isolate Number	Species	Colony concentration (cells per ml)	Source
1	MK15	*Dickeya solani*	10^3	River Water, Scotland
2	RW192/1	*Dickeya zeae*	10^7	River water, England
3	Blank			
4	MK13	*Dickeya solani*	10^3	Rotten tuber, Israel
5	MK15	*Dickeya solani*	10^7	River water, Scotland
6	MK13	*Dickeya solani*	10^7	Rotten tuber, Israel
7	Blank			
8	PRI3327	*Dickeya dianthicola*	10^7	Potato tuber, England
9	NCCPB549	*Pectobacterium atrosepticum*	10^7	Potato tuber, England
10	CSL20621674	*Dickeya solani*	10^7	*Hyacinthus* sp., England

Table 2.5. ECH Primers and Probes Sequences Used in "Ring Test"

ECH	Forward primer (5'-3')	GAGTCAAAAGCGTCTTGCGAA
	Reverse primer (5'-3')	CCCTGTTACCGCCGTGAA
	Probe* (5'-3')	CTGACAAGTGATGTCCCCTTCGTCT AGAGG

*Probe is labelled with 6-FAM

Laboratories were advised to use the sap directly and initially test the sap by plating onto CVPM and incubating at 36°C for 48 hours and to enumerate pit-forming colonies at various dilutions: neat, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} and test resultant colonies with the ECH assay (Diallo *et al.*, 2009) to ensure the presence of *Dickeya* sp. The details of the conventional PCR 'Nassar assay' (Nassar *et al.*, 1996) were also included. Once the initial steps were carried out the sap samples were to be tested directly by taking 20µl and boiling at 100°C for 5min. In the PCR reactions, 1µl of the boiled cells

was used. Extracted DNA was also tested in the real-time PCR reactions. Two hundred and fifty microliters of the 10 sap samples was enriched in 250µl of PEM and the samples incubated for at least 48 hours at 36ºC. The enriched samples were tested both directly by taking 20µl and boiling at 100ºC for 5min and 1µl of boiled cells used in the PCR assays. DNA was also extracted from the enriched samples and 5µl of extract used in the PCR assays.

2.3 Results from Real-time Diagnostic Assays

2.3.1 *Results from Initial Evaluation of the SOL-C and* fusA *Assays using Strains from the SASA Collection*

In order to test the effectiveness of two separate real-time PCR assays (*fusA* and SOL-C) specific for the detection of *Dickeya solani* 73 isolates from the genera *Dickeya* and *Pectobacterium* were tested. This collection comprised of 21 isolates of *D. solani* collected from a variety of sources, 39 reference isolates from the other *Dickeya* species and 13 reference isolates of *Pectobacterium*. The results from this preliminary evaluation are found in Tables 2.6, 2.7, 2.8 and 2.9. A positive result for *D. solani* was recorded for a C_t value of less than 25 and those above 25 or undetermined were recorded as negative. Results that are identified as undetermined were analysed and likely to have fallen under the detection limit of the assay.

Table 2.6 C_t Values of *Dickeya solani* Isolates Using *fusA* and SOL-C Assays

Strain	Attributed species	C_t values obtained in real-time qPCR assays			
		fusA	*fusA*	SOL-C	SOL-C
MK8	*Dickeya solani*	37.3	33.4	35.7	Undetermined
MK10	*D. solani*	17.8	17.3	15.3	15.5
MK11	*D. solani*	17.7	Undetermined	14.9	16.4
MK12	*D. solani*	18.3	17.1	14.3	16.7
MK13	*D. solani*	Undetermined	20.0	17.1	16.9
MK14	*D. solani*	18.6	18.1	17.3	16.6
MK15	*D. solani*	32.8	32.8	30.9	31.1
MK16	*D. solani*	18.3	15.5	14.1	16.4
CSL20621674	*D. solani.*	Undetermined	Undetermined	Undetermined	Undetermined
A101/9	*D. solani*	18.4	17.7	15.9	16.7
A101/10	*D. solani*	17.9	17.5	15.7	16.3
A101/11	*D. solani*	19.4	18.8	18.0	17.3
A101/12	*D. solani*	18.1	18.3	16.2	18.3
B2744	*D. solani*	19.1	17.7	15.3	17.1
B2745	*D. solani*	19.0	19.4	16.7	17.4
B1	*D. solani*	20.2	Undetermined	19.0	18.7
DM157	*D. solani*	21.3	Undetermined	17.8	22.0
DM159	*D. solani*	18.4	18.7	14.2	18.7
6395	*D. solani*	14.4	16.3	10.5	15.0
6396	*D. solani*	17.9	16.3	15.1	15.6
CSL20710504	*D. solani*	16.9	17.7	14.6	16.1

Undetermined as identified by real-time PCR analysis programme

Table 2.7 C_t Values of *Dickeya* spp. Isolates Using *fusA* and SOL-C Assays

Strain	Attributed species	C_t values obtained in real-time qPCR assays			
		fusA	*fusA*	SOL-C	SOL-C
MK1	*Duc3**	39.1	32.0	Undetermined	17.4
MK2	*Duc3**	19.9	19.6	15.7	18.2
MK3	*Duc3**	Undetermined	Undetermined	Undetermined	Undetermined
MK4	*Duc3**	Undetermined	Undetermined	Undetermined	Undetermined
MK5	*Duc3**	Undetermined	Undetermined	Undetermined	Undetermined
MK6	*Duc3**	Undetermined	35.5	Undetermined	Undetermined
MK7	*Duc3**	Undetermined	Undetermined	Undetermined	Undetermined
MK9	*Duc3**	Undetermined	Undetermined	Undetermined	Undetermined
MK17	*Dickeya zeae*	Undetermined	9.4	Undetermined	Undetermined
MK18	*D. zeae*	34.5	15.0	Undetermined	35.9
MK19	*D. zeae*	28.6	27.6	23.9	26.6
MK20	*D. zeae*	38.4	Undetermined	35.4	36.6
CSL20708100	*D. dianthicola*	28.9	28.6	28.5	24.5
CSL20714261	*Duc2**	Undetermined	Undetermined	35.6	Undetermined
CSL20714521	*D. dianthicola*	35.5	34.5	Undetermined	Undetermined
RW 192/1	*D. zeae*	Undetermined	Undetermined	Undetermined	Undetermined
RW 240/1	*Dickeya* sp.	37.2	26.6	36.7	25.2
402	*D. chrysanthemi*	36.1	35.2	33.2	Undetermined
453	*D. dianthicola*	Undetermined	Undetermined	Undetermined	Undetermined
454	*D. dadantii*	Undetermined	Undetermined	Undetermined	Undetermined
516	*D. chrysanthemi*	Undetermined	Undetermined	Undetermined	Undetermined
569	*Dickeya* sp.	35.9	35.0	Undetermined	34.8
898	*D. dadantii*	32.9	32.2	34.4	32.7

Strain	Attributed species	C_t values obtained in real-time qPCR assays			
		fusA	*fusA*	SOL-C	SOL-C
1121	*D. zeae*	17.9	18.8	15.1	17.3
1385	*D. dianthicola*	35.1	36.9	34.2	33.6
1861	*D. chrysanthemi*	33.2	Undetermined	26.8	32.0
2260	*D. dianthicola*	36.4	36.3	35.1	35.7
2339	*D. zeae*	Undetermined	Undetermined	Undetermined	Undetermined
2511	*D. paradisiaca*	Undetermined	Undetermined	Undetermined	Undetermined
2538	*D. zeae*	Undetermined	Undetermined	37.0	Undetermined
2541	*D. zeae*	36.9	Undetermined	34.5	35.7
2546	*D. zeae*	34.0	31.5	30.0	32.4
2976	*D. dieffenbachiae*	34.4	Undetermined	36.3	37.4
3274	*Duc3**	Undetermined	Undetermined	Undetermined	Undetermined
3531	*D. zeae*	35.3	17.0	36.1	36.3
3532	*D. zeae*	Undetermined	34.4	33.4	35.3
3533	*D. chrysanthemi*	38.0	Undetermined	37.1	35.3
3534	*D. dianthicola*	Undetermined	Undetermined	36.4	Undetermined

*Duc2 and Duc3 as described by Parkinson *et al.*, 2009.

Undetermined as described by real-time PCR analysis programme

Table 2.8 C_t Values of Other Species Isolates Using *fusA* and SOL-C Assays

Strain	Attributed species	C_t values obtained in real-time qPCR assays			
		fusA	*fusA*	SOL-C	SOL-C
312	*Pectobacterium carotovorum*	*Undetermined*	Undetermined	37.0	Undetermined
549	*P. atrosepticum*	Undetermined	26.6	25.7	Undetermined
1092	*Erwinia chrysanthemi*	35.4	30.4	33.2	32.9
1578	*P. rhapontici*	37.5	32.1	Undetermined	Undetermined
2264	*E. chrysanthemi*	34.8	Undetermined	Undetermined	36.5
2265	*E. chrysanthemi*	Undetermined	Undetermined	34.5	Undetermined
2295	*Pantoea stewartii* subsp. *stewartii*	Undetermined	Undetermined	Undetermined	Undetermined
2795	*P. betavasculorum*	34.1	Undetermined	Undetermined	Undetermined
2971	*E. herbicola*	34.0	33.2	32.5	32.1
3004	*E. cypripedii*	31.3	32.7	29.5	30.6
3701	*Dickeya wasabiae*	Undetermined	35.9	Undetermined	Undetermined
3839	*D. carotovorum* subsp. *odoriferum*	Undetermined	36.0	Undetermined	35.1
6397	*D. chrysanthemi*	34.5	35.3	30.9	33.7

Undetermined as described by real-time PCR analysis programme

Table 2.9 Results from *fusA* and SOL-C Evaluation Using SASA Reference Strains

	fusA (A)	*fusA* (B)	SOL-C (A)	SOL-C (B)
D. solani detected	18	18	18	18
D. solani not detected	3	3	3	3
Dickeya sp. detected as *D. solani*	2	2	2	2
Other species detected as *D. solani*	0	0	0	0

The preliminary evaluation of both the *fusA* and SOL-C assays delivered similar results, with both assays correctly identifying 18 out of the 21 *Dickeya solani* strains studied here. Only two strains out of 52 of non-*Dickeya solani* strains: MK2 and NCPPB1121, gave false positive results. MK2, is a *Dickeya* isolate assigned to a new clade, Duc3 and NCPPB1121 was isolated from pineapple in 1962 and identified as *D. zeae* based on *recA* sequencing, as described in Appendix Table A.1 (Parkinson *et al.*, 2009). The *D. solani* strains that were falsely identified as negative were MK8, MK15, and CSL20621674. MK8 and MK15 were both isolated from the same river in Scotland; MK8 was isolated in 2007 and MK15 isolated in 2008. CSL20621674 was isolated from hyacinth in England in 2007. In general the SOL-C assay gave predominantly lower C_t values when compared to *fusA*. Assays were also conducted in duplicate using fresh samples of boiled cells each time and although overall good reproducibility for each assay was observed there were a number of conflicting results between the duplicates, specifically MK1 and MK18 with SOL-C and MK11, MK13, MK17, MK18, B1 and 3531 with the *fusA* assay.

2.3.2 *Results from Initial Evaluation of the SOL-C and* fusA *Assays using Strains from the EUPHRESCO Collection*

A further evaluation was conducted with both assays on a second collection of strains this time assembled as part of a EUPHRESCO project (http://euphresco.net) exploring the diversity of *Dickeya* spp. across Europe. This collection comprised of 11 isolates of *D. solani* collected from a variety of sources, and 25 reference isolates from the other *Dickeya* species. The results from this evaluation are found in Tables 2.10 and 2.11. The limits for C_t values were as described above. Using the EUPHRESCO strains, 10 of the 11 *D. solani* strains were correctly identified, with only isolate PRI3296 not detected,

and only one false positive: PRI3328, previously identified as *D. dianthicola,* was observed. The details of the strains can be found in Appendix Table A.1.

Table 2.10 C_t Values of EUPHRESCO Reference Strains Using *fusA* and SOL-C Assays

Strain	Attributed species	C_t values obtained in real-time qPCR assays	
		fusA	SOL-C
2019	*D. solani*	14.3	16.8
2187	*D. solani*	13.2	14.7
2222	*D. solani*	13.7	16.6
2276	*D. solani*	13.6	16.3
3228	*D. solani*	15.1	14.9
3239	*D. solani*	14.7	16.7
3294	*D. solani*	14.3	15.1
3295	*D. solani*	16.2	16.5
3296	*D. solani*	Undetermined	Undetermined
3336	*D. solani*	15.8	15.8
3337	*D. solani*	17.6	16.6
980	*D. dianthicola*	35.3	Undetermined

Strain	Attributed species	C_t values obtained in real-time qPCR assays	
		fusA	SOL-C
1259	*D. dieffenbachiae*	34.7	30.0
2114	*D. dianthicola*	Undetermined	Undetermined
2115	*D. dianthicola*	Undetermined	Undetermined
2116	*D. dianthicola*	30.0	35.6
2117	*D. chrysanthemi*	Undetermined	Undetermined
2118 (402)	*D. chrysanthemi* pathovar *chrysanthemi*	Undetermined	35.7
2119	*D. chrysanthemi* pathovar *chrysanthemi*	Undetermined	Undetermined
2120	*D. dadantii*	Undetermined	36.9
2121 (1121)	*D. dadantii*	Undetermined	Undetermined
2122	*D. dadantii*	Undetermined	Undetermined
2124	*D. dieffenbachiae*	Undetermined	Undetermined
2125 (2976)	*D. dieffenbachiae*	Undetermined	37
2126	*D. dieffenbachiae*	Undetermined	Undetermined

Strain	Attributed species	C_t values obtained in real-time qPCR assays	
		fusA	SOL-C
2127	*D. paradisiaca*	Undetermined	34.5
2129 (2511)	*D. paradisiaca*	Undetermined	Undetermined
2131 (2538)	*D. zeae*	Undetermined	Undetermined
2132 (2339)	*D. zeae*	Undetermined	Undetermined
2133	*D. zeae*	Undetermined	Undetermined
3327 (3528)	*D. dianthicola*	30.8	37.6
3328 (3530)	*D. dianthicola*	16.2	13.1
3329 (3531)	*D. zeae*	Undetermined	Undetermined
3330 (3533)	*D. chrysanthemi*	Undetermined	Undetermined
3332 (3237)	*D. dadantii*	Undetermined	Undetermined
3334 (3344)	*D. dianthicola*	Undetermined	36.6

*Undetermined as identified by real-time PCR analysis programme

Table 2.11 Results from *fusA* and SOL-C Evaluation Using EUPHRESCO Reference Strains

	fusA	SOL-C
D. solani detected	10	10
D. solani not detected	1	1
Dickeya sp. detected as *D. solani*	1	1

2.3.3 *Results from the Evaluation of Assays Using a 'Ring Test'*

Details of the method used and the partners involved in the ring test are given in section 2.2.4. Each institute was provided with primer pairs and recommended temperature programmes with the advice that the institute's own equipment, buffers, *Taq* polymerase and DNA extraction method be used. Details of the DNA extraction methods used by each institute were not provided. It was also advised that each institute carry out an initial conventional PCR assay (Nassar *et al.*, 1996) to assess whether *Dickeya* sp. was present in the samples. Only two institutes acknowledged they had/had not used the 'Nassar Assay,' with Institute One not carrying out the assay and Institute Two achieving the same results when the 'Nassar Assay' was carried out using boiled cells as the DNA template and using DNA extracts. Institute Two did not detect *Dickeya* sp. in the blank samples or the *Pectobacterium atrosepticum* sample using the 'Nassar Assay' but also did not detect *Dickeya* sp. in the low concentrations of the MK13 and MK15 samples. *Dickeya* sp. was detected in the higher concentrations of MK13 and MK15 and also the *D. solani* isolate CSL20621674.

Table 2.12 Results of 'Nassar Assay' from Institute Two on Unenriched Samples

Strain	Species	Boiled cells	DNA extract
MK15 L*	*D. solani*	Negative	Negative
RW192/1	*D. zeae*	Positive	Positive
Blank		Negative	Negative
MK13 L*	*D. solani*	Negative	Negative
MK15 H*	*D. solani*	Positive	Positive
MK13 H*	*D. solani*	Positive	Positive
Blank		Negative	Negative
3327	*D. dianthicola*	Positive	Positive
549	*P. atrosepticum*	Negative	Negative
20621674	*D. solani*	Positive	Positive

*L refers to low concentrations of sample and H refers to high concentrations.

Table 2.13 Results of 'Nassar Assay' from Institute Two on Enriched Samples

Strain	Species	Boiled cells	DNA extract
MK15 L*	*D. solani*	Negative	Negative
RW192/1	*D. zeae*	Positive	Positive
Blank		Negative	Negative
MK13 L*	*D. solani*	Negative	Negative
MK15 H*	*D. solani*	Positive	Positive
MK13 H*	*D. solani*	Positive	Positive
Blank		Negative	Negative
3327	*D. dianthicola*	Positive	Positive
549	*P. atrosepticum*	Negative	Negative
20621674	*D. solani*	Positive	Positive

*L refers to low concentrations of sample and H refers to high concentrations.

In this study, C_t values of less than 30 were considered to positively identify *Dickeya solani*. Similar results were seen from all six institutes when the assays were carried out using boiled cells. As shown in Table 2.13, the unenriched samples, MK13 and MK15,

in high concentrations, and CSL20621674 were correctly identified as *D. solani* by all six institutes. Using unenriched samples, five institutes correctly identified MK13 and MK15 in high concentrations and CSL20621674 as *D. solani*. When DNA extract samples were used, results were more variable.

Results of the Evaluation of Assays from Institute One

Table 2.14 Results from Institute One for Assessing the Reproducibility and Sensitivity of the Real-Time PCR Assays *fusA* and SOL-C.

		Unenriched boiled cells	Enriched boiled cells	Unenriched DNA (A)	Enriched DNA (A)	Unenriched DNA (B)	Enriched DNA (B)
fusA	*D. solani* correctly detected	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	0	0	3 MK13H MK15H CSL20621674
	D. solani not detected	2 MK13L MK15L	2 MK13L MK15L	2 MK13L MK15L	5	5	2 MK13L MK15L
	Other species giving false positives results as *D. solani*	0	0	0	0	0	0
SOL-C	*D. solani* correctly detected	0	0	3 MK13H MK15H CSL20621674	0	3 MK13H MK15H CSL20621674	0
	D. solani not detected	5	5	2 MK13L MK15L	5	2 MK13L MK15L	5
	Other species detected as *D. solani*	0	0	0	0	0	0

H indicates higher concentration of the bacterial suspension and L indicates lower concentration of the bacterial suspension

Breakdown of Results from Institute One (Table 2.14)

Results from fusA *Assay*

In addition to using boiled cells as the DNA template for assessing the two real-time PCR assays, Institute One used two other DNA extraction methods, however, details of these methods were not provided. When using unenriched and enriched boiled cells as the DNA template, the higher concentrations of MK13 and MK15 and CSL20621674 were correctly and consistently identified as *Dickeya solani*. Using DNA extraction method A on unenriched samples, the higher concentrations of MK13 and MK15 and CSL20621674 were correctly identified as *D. solani*. When extraction method A was used on enriched samples *D. solani* was not detected. Using the DNA extraction method B on unenriched samples, none of the *D. solani* isolates were detected. When extraction method B was applied to enriched samples, the higher concentrations of MK13 and MK15 and CSL20621674 were correctly identified. No false positives occurred.

Results from SOL-C Assay

Using boiled cells as the template for the SOL-C assays did not detect any of the *Dickeya solani* isolates regardless of whether the samples had been enriched or not. DNA extraction (A and B) of unenriched samples, regardless of method, correctly detected higher concentrations of MK13 and MK15 and CSL20621674 but not the lower concentrations of MK13 and MK15. When the extraction methods were applied to enriched samples none of the *D. solani* isolates were detected.

From these results, there is little difference in qualitatively identifying the presence of *Dickeya solani* detection between unenriched and enriched samples and between DNA extraction methods A and B and boiled cells. Institute One did not carry out PCR assays such as the ‘Nassar Assay’ or ECH Assay to detect whether *Dickeya* sp. were present in any of the samples sent.

Results of the Evaluation of Assays from Institute Two

Table 2.15 Results from Institute Two for Assessing the Reproducibility and Sensitivity of the Real-Time PCR Assays *fusA* and SOL-C.

		Unenriched boiled cells	Enriched boiled cells	Unenriched DNA	Enriched DNA
fusA	*D. solani* correctly detected	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	2 MK13H CSL20621674	1 CSL20621674
	D. solani not detected	2 MK13L MK15L	2 MK13L MK15L	3 MK13L MK15L MK15H	4
	Other species giving false positive results as *D. solani*	1 PRI3327	0	0	0
SOL-C	*D. solani* correctly detected	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	2 MK15H CSL20621674
	D. solani not detected	2 MK13L MK15L	2 MK13L MK15L	2 MK13L MK15L	3 MK13L MK13H MK15L
	Other species giving false positive results as *D. solani*	1 PRI3327	1 PRI3327	1 PRI3327	1 PRI3327

H indicates higher concentration of the bacterial suspension and L indicates lower concentration of the bacterial suspension

Breakdown of Results from Institute Two (Table 2.15)

Results from fusA *Assay*

The results from Institute Two showed that both unenriched and enriched boiled cells gave the same outcome with both preparations positively identifying MK13 and MK15 at higher concentrations and CSL20621674 as *Dickeya solani*. , PRI3327, *D. dianthicola*, was incorrectly identified as *D. solani* in unenriched boiled cells. Using unenriched DNA extracts, two samples were correctly identified as *D. solani*: CSL20621674 and the high concentration of MK13; the DNA extracts from enriched samples, only positively identified CSL20621674.

Results from SOL-C Assay

When using the SOL-C assay, Institute Two correctly identified three *Dickeya solani* samples from unenriched boiled cells: the higher concentrations of MK13 and MK15 and CSL20621674. When enriched boiled cells were used as the DNA template, the same results were seen as with the unenriched cells. The same results were also seen when DNA extractions were used taken from unenriched cells and higher concentrations of MK13 and MK15 and CSL20621674 were correctly identified. When DNA extracts were taken from enriched samples, the higher concentration of MK15 and CSL20621674 were correctly identified as *D. solani*. In all combinations of unenriched or enriched boiled cells or DNA extracts, PRI3327 was incorrectly identified as *D. solani*.

Results of the Evaluation of Assays from Institute Three

Table 2.16 Results from Institute Three for Assessing the Reproducibility and Sensitivity of the Real-Time PCR Assays *fusA* and SOL-C.

		Unenriched boiled cells	Enriched boiled cells	Unenriched DNA	Enriched DNA
fusA	*D. solani* correctly detected	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674
	D. solani not detected	2 MK13L MK15L	2 MK13L MK15L	2 MK13L MK15L	2 MK13L MK15L
	Other species giving false positive results as *D. solani*	0	0	0	0
SOL-C	*D. solani* correctly detected	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	4 MK13L MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674
	D. solani not detected	2 MK13L MK15L	2 MK13L MK15L	1 MK15L	2 MK13L MK15L
	Other species giving false positive results as *D. solani*	0	0	1 NCCPB549	0

H indicates higher concentration of the bacterial suspension and L indicates lower concentration of the bacterial suspension

Breakdown of Results from Institute Three (Table 2.16)

Results from fusA *Assay*

The *fusA* results from Institute Three were the same regardless of sample preparation or DNA extraction and high concentrations of MK13 and MK15 and CSL20621674 were correctly identified as *Dickeya solani*.

Results from SOL-C Assay

For the SOL-C assay for both unenriched and enriched boiled cells higher concentrations of MK13 and MK15 and CSL20621674 were correctly identified as *Dickeya solani*. When unenriched DNA extracts were used, both concentrations of MK13, higher concentrations of MK15 and CSL20621674 were correctly identified as *D. solani* but NCCPB549 (*Pectobacterium atrosepticum)* was incorrectly identified as *D. solani*.. When enriched DNA extracts were used higher concentrations of MK13 and MK15 and CSL20621674 were correctly identified as *D. solani*.

Results of the Evaluation of Assays from Institute Four

Table 2.17 Results from Institute Four for Assessing the Reproducibility and Sensitivity of the Real-Time PCR Assays *fusA* and SOL-C.

		Unenriched boiled cells	Enriched boiled cells	Unenriched DNA	Enriched DNA
fusA	*D. solani* correctly detected	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674
	D. solani not detected	2 MK13L MK15L	2 MK13L MK15L	2 MK13L MK15L	2 MK13L MK15L
	Other species giving false positive results as *D. solani*	0	0	0	1 Blank control
SOL-C	*D. solani* correctly detected	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674
	D. solani not detected	2 MK13L MK15L	2 MK13L MK15L	2 MK13L MK15L	2 MK13L MK15L
	Other species giving false positive results as *D. solani*	0	0	0	0

H indicates higher concentration of the bacterial suspension and L indicates lower concentration of the bacterial suspension

Breakdown of Results from Institute Four (Table 2.17)

Results from fusA *Assay*

Institute Four correctly identified higher concentrations of MK13 and MK15 and CSL20621674 as *Dickeya solani* from both enriched and unenriched boiled cell samples. When DNA extract samples were used, the unenriched samples of higher concentrations of MK13 and MK15 and CSL20621674 were correctly identified. When enriched DNA extracts were used the same three samples were correctly identified, however one of the blank controls was also identified as *D. solani*.

Results from SOL-C Assay

The results from SOL-C were the same regardless of the enrichment or DNA extraction. Under all test conditions, higher concentrations of MK13 and MK15 and CSL20621674 were correctly identified as *Dickeya solani* and there were no false positives.

Results of the Evaluation of Assays from Institute Five

Table 2.18 Results from Institute Five for Assessing the Reproducibility and Sensitivity of the Real-Time PCR Assays *fusA* and SOL-C.

		Unenriched boiled cells	Enriched boiled cells	Unenriched DNA	Enriched DNA
fusA	*D. solani* correctly detected	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	0	0
	D. solani not detected	2 MK13L MK15L	2 MK13L MK15L	5	5
	Other species giving false positive results as *D. solani*	0	0	0	1 NCCPB549
SOL-C	*D. solani* correctly detected	3 MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	0	0
	D. solani not detected	2 MK13L MK15L	2 MK13L MK15L	5	5
	Other species giving false positive results as *D. solani*	0	0	0	0

H indicates higher concentration of the bacterial suspension and L indicates lower concentration of the bacterial suspension

Breakdown of Results from Institute Five (Table 2.18)

Results from fusA *Assay*

The *fusA* assay results from Institute Five show the correct identification of higher concentrations of MK13 and MK15 and CSL20621674 using both enriched and unenriched boiled cells. When DNA extracts were used none of the *Dickeya solani* samples were correctly identified. When enriched DNA extracts were used sample NCCPB549, *Pectobacterium atrosepticum*, was incorrectly identified as *D. solani*.

Results from SOL-C Assay

Using the SOL-C assay, higher concentrations of MK13 and MK15 and CSL20621674 were correctly identified from both unenriched and enriched boiled cells, and using DNA extracts, no samples were identified as *Dickeya solani*.

Results of the Evaluation of Assays from Institute Six

Table 2.19 Results from Institute Six for Assessing the Reproducibility and Sensitivity of the Real-Time PCR Assays *fusA* and SOL-C.

		Unenriched boiled cells	Enriched boiled cells	Unenriched DNA	Enriched DNA
fusA	*D. solani* correctly detected	3 MK13H MK15H CSL20621674	4 MK13L MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	4 MK13L MK13H MK15H CSL20621674
	D. solani not detected	2 MK13L MK15L	1 MK15L	2 MK13L MK15L	1 MK15L
	Other species giving false positive results as *D. solani*	0	0	0	0
SOL-C	*D. solani* correctly detected	3 MK13H MK15H CSL20621674	4 MK13L MK13H MK15H CSL20621674	3 MK13H MK15H CSL20621674	4 MK13L MK13H MK15H CSL20621674
	D. solani not detected	2 MK13L MK15L	1 MK15L	2 MK13L MK15L	1 MK15L
	Other species giving false positive results as *D. solani*	0	0	1 NCCPB549	0

H indicates higher concentration of the bacterial suspension and L indicates lower concentration of the bacterial suspension

Breakdown of Results from Institute Six (Table 2.19)

Results from fusA *Assay*

Institute Six results for the *fusA* assay suggest that enriching the sample provided better identification of *Dickeya solani* regardless of whether DNA extracts or boiled cells were used. When samples were enriched, lower concentrations of MK13 were correctly identified as *D. solani*, although the C_t value was 29.9 which is close to the limit of 30, in addition to higher concentrations of MK13 and MK15 and CSL20621674 correctly identified. When unenriched boiled cells and DNA extracts were used, higher concentrations of MK13 and MK15 and CSL20621674 were correctly identified as *D. solani*.

Results from SOL-C Assay

The results for the SOL-C assay produced the same results as for *fusA*, with unenriched boiled cells and DNA extracts correctly identifying higher levels of MK13 and MK15 and CSL20621674. When samples were enriched, both lower and higher concentrations of MK13 were correctly identified as well as higher concentrations of MK15 and CSL20621674 were also correctly identified. Unenriched DNA extracts incorrectly identified NCCPB549 (*Pectobacterium atrosepticum*) as *D. solani*.

Results of the Evaluation of Assays Based on Samples Correctly Identified in Using the ECH Assay

Included with the primers for the *Dickeya solani* specific real-time assays, were the primers and method for detecting *Dickeya* sp. using real-time PCR and the primer pair ECH/ECH' (Diallo *et al.*, 2009) with the request that the institutes carry out the assessment to detect whether *Dickeya* sp. were present in the samples provided. It was suggested that the ECH assay be carried out on the same sample preparations as the other two real-time assays, i.e., using unenriched and enriched boiled cells as the DNA template and DNA extracts from unenriched and enriched cells. The total number of samples of strains used varied, depending on the results from the ECH assay results carried out by the institutes as shown in Table 2.20. Institute One did not carry out these detection tests and their results have been disregarded in further analysis of these results. Clearly results suggest that at lower concentrations both *fusA* and SOL-C assays lacked the necessary sensitivity, therefore concentrating solely on the higher concentrations of MK13 and MK15 and CSL20621674, as shown in Table 2.21 below, both assays produce consistent detection of *D. solani* when carried out by other institutes.

Table 2.20 Results of Assessment of Two Real-Time PCR Assays Based on ECH Assay Results for *Dickeya* sp.

	Institute One	Institute Two	Institute Three	Institute Four	Institute Five	Institute Six
Unenriched boiled cells	N/T	5 MK13H MK15H RW192/1 PRI3327 CSL20621674	5 MK13H MK15H RW192/1 PRI3327 CSL20621674	5 MK13H MK15H RW192/1 PRI3327 CSL20621674	5 MK13H MK15H RW192/1 PRI3327 CSL20621674	5 MK13H MK15H RW192/1 PRI3327 CSL20621674
Enriched boiled cells	N/T	5 MK13H MK15H RW192/1 PRI3327 CSL20621674	5 MK13H MK15L MK15H RW192/1 CSL20621674	7	6 MK13H MK15L MK15H RW192/1 PRI3327 CSL20621674	7
Unenriched DNA extracts	N/T	4 MK13H MK15H RW192/1 CSL20621674	5 MK13H MK15H RW192/1 PRI3327 CSL20621674	5 MK13H MK15H RW192/1 PRI3327 CSL20621674	5 MK13H MK15H RW192/1 PRI3327 CSL20621674	5 MK13H MK15H RW192/1 PRI3327 CSL20621674
Enriched DNA extracts	N/T	5 MK13H MK15H RW192/1 PRI3327 CSL20621674	N/A	7	4 MK13H MK15H RW192/1 PRI3327	6 MK13H MK15L MK15H RW192/1 PRI3327 CSL20621674

N/T – Not tested N/A – Not accepted as negative control failed.

Summary of Results from 'Ring Test'

Table 2.21 Summary Results of the Assessment of Real-Time Assays *fusA* and SOL-C Specific for *Dickeya solani* with Correctly Identified Samples.

	fusA		SOL-C	
	Correctly identified as *D. solani*	Incorrectly identified as *D. solani*	Correctly identified as *D. solani*	Incorrectly identified as *D. solani*
Unenriched boiled cells	15/15	1/15	14/15	1/15
Enriched boiled cells	13/15	0/14	15/15	1/13
Unenriched DNA	11/13	0/12	12/13	1/12
Enriched DNA	4/7	1/9	8/10	1/12

A summary of the identification of *Dickeya solani* samples from the six institutes is presented in Table 2.21. Based on these results there does not appear to be a difference between the sensitivity when unenriched or enriched boiled cells are used., There is more variability when DNA extracts are used, and unenriched DNA extracts gave more consistent results. The DNA extraction method was not uniform between the Institutes.

Results Assessing the* fusA *Assay

The unenriched boiled cells used with the *fusA* assay correctly identified all three of the *Dickeya solani* isolates at each of the Institutes 2-6, although PRI3327 (*D. dianthicola*) was incorrectly identified as *D. solani* by Institute Two. Using enriched DNA extracts with the *fusA* assay allowed for the correct detection of 13 out of the 15 *Dickeya solani* isolates, with higher concentrations of MK15 not detected by Institute Two and Institute Five not detecting the higher concentrations of MK13. When enriched DNA extracts were used with *fusA*, four out of the seven *D. solani* isolates were correctly identified, with Institute Two detecting false negatives for the high concentrations of both MK13 and MK15 and Institute Five also incorrectly identifying the higher concentration of MK13 as negative.

Results Assessing the SOL-C Assay

When unenriched boiled cells were used as the DNA template for the SOL-C assay, 14 out of 15 *Dickeya solani* isolates were correctly identified, the only isolate not detected was the lower concentration of MK13 used by Institute Two, and Institute Two also incorrectly detected PRI3327 (*D. dianthicola*) as *D. solani*. When enriched boiled cells were used, all 15 *D. solani* isolates were identified; however, as with the unenriched boiled cells, Institute Two incorrectly identified PRI3327 as *D. solani*.

When DNA extracts were used as the DNA template, 12 out of 13 of *Dickeya solani* isolates were correctly identified when the extractions were from unenriched samples and only one isolate, NCCPB549 (*Pectobacterium atrosepticum*) was incorrectly identified as *D. solani* by Institute Six. Institute Five did not detect the higher concentration of MK13 as *D. solani*. When the DNA was extracted from enriched samples, eight out of the ten *D. solani* isolates were correctly identified, with false negatives found for the higher concentrations of MK13 and MK15 by Institute Five, and Institute Five incorrectly identified PRI3327 as *D. solani.*

There is a correlation between the false positives and false negatives in the assays. Institute Two incorrectly detected PRI3327 (*D. dianthicola*) as *D. solani* in three out of the four tests, using unenriched boiled cells for the template with *fusA* and using both unenriched and enriched DNA extracts for SOL-C. CSL20621674 was correctly identified by all institutes and under all sample preparations. Only Institute Four correctly identified all samples; however, the results for using enriched boiled cells and *fusA* was discounted as the negative control sample gave a C_t value of 25.9 and therefore a false positive. The results from Institute Three testing SOL-C with enriched DNA extracts was also discounted as a negative control had a C_t value of 29.9 when tested using the ECH assay. Institute One did not test for *Dickeya* sp. in the samples prior to carrying out the *fusA* and SOL-C assays therefore the results were not included in the overall analysis of the reproducibility and reliability of the real-time assays.

2.4 Discussion of Results from Design of Real-Time Diagnostic Assays Specific for *Dickeya solani*

The soft rot enterobacteriaceae *Pectobacterium* spp. and *Dickeya* spp. are important pathogens of potato and other crops and can affect the growing plant through expression of blackleg or cause soft rot in tubers during storage, both of which reduce yield and quality (Mansfield *et al.*, 2012). Seed classification systems were introduced in virtually every country producing seed potatoes in order to ensure the high quality of propagation material and these schemes set the tolerance levels for soft rot and blackleg (Czajkowski *et al*., 2015). The tolerances for blackleg in European seed classification vary from country to country as there is no uniform policy in the European Union (Toth *et al.*, 2011). Efficient and cost-effective detection and identification methods are essential to investigate the ecology and pathogenesis of the *Dickeya* spp. as well as improving seed classification systems (Czajkowski *et al*., 2015).

Detection and identification of plant pathogenic bacteria previously relied on the isolation on selective media and subsequent identification through biochemical assays but isolation of pathogens from plant material was not guaranteed (Pérombelon, 2002). Advancements in molecular techniques have facilitated the development of new diagnostic techniques based on DNA/nucleic acid analysis. PCR assays have been increasingly used for the specific detection and identification of the *Dickeya* genus with the most widely used based on the ADE primers (ADE1 and ADE2) from the pectate lyase gene (*pel*) although others have been designed for the detection of soft rot erwiniae as a single group (Nassar *et al.*, 1996; Toth *et al.*, 1999; Toth *et al.*, 2001). Such methods enable the detection of pathogens even when population numbers are low or when there is a mix of closely related pathogens. In the case of *Dickeya solani*, advancements in molecular techniques have also aided taxonomic classification (van der Wolf *et al.*, 2014). Whole genome sequencing has become a cost-effective and efficient method for the identification and classification of plant pathogens and genomics provides a solid platform for the development of taxonomy and for deriving a detailed knowledge of gene functions and structures, specifically pathogenesis and virulence and interactions with the host and other organisms (Konstantinidis *et al.*, 2006; Demuth *et al.*, 2008; Chun and Rainey, 2014; Pritchard *et al.*, 2016). Genomic information can also be used in ecological studies to understand the spread, establishment and survival of bacterial pathogens in given environments and the overall investigation of plant

pathogenic bacteria relies on gathering information that can be used to develop new disease control strategies to protect crops against bacterial diseases (Wilson, 2012).

The currently accepted methods for the detection and identification of *Dickeya solani* rely on the isolation and testing of viable cells from environmental samples. In many cases, an enrichment step is necessary to increase the densities of *Dickeya* spp. in the samples before isolation of the bacteria, DNA extraction and once positively identified using the Nasser assay, the sample is sequenced based on the *recA* gene and identified based on differences within this gene (Pérombelon and van der Wolf, 2002; Waleron *et al.*¸2002; Parkinson *et al.,* 2009). Whilst the *recA* sequencing method provides a convenient means of identifying strains to a species and subspecies level, it is costly and time consuming especially when sampling numbers are high as is required for seed potato certification schemes. In Scotland, where there is a zero tolerance for *Dickeya* spp., visual inspections are carried out twice during the growing season for symptoms of blackleg/soft rot disease and any seed tubers that are brought into the country must be tested for quarantine diseases including brown rot, ring rot and *Dickeya* spp. These tests can take up to two weeks which causes inconvenience to the growers (SASA, 2011). The use of molecular methods overcomes many of these current limitations because they do not require living cells, remove the requirement to culture, are highly specific and reproducible and provide fast qualitative and quantitative detection of bacteria even when there are other bacteria present (Rastogi and Sani, 2011). The methods designed in this project amplified a specific target DNA sequence using PCR and these methods rely on the template DNA to be extracted from previously isolated bacterial colonies, complex environmental samples or from mixed cultures obtained by enrichment. The specificity of the PCR reaction depends on the primer pair used. By using real-time PCR the results on the amplification can be seen in real-time, rather than waiting for post-amplification manipulation to visualise results such as gel electrophoresis. In our studies we designed the primers based on two different genes: *fusA* and SOL-C, designed using the biostatistical pathway designed by Pritchard *et al.* (2013).

The two real-time assays developed in this study suggest that they can be utilised as the sole diagnostic test specifically for *D. solani* which would enable the detection and diagnostic process to be quickened and would reduce costs. The preliminary evaluation carried out at SASA showed that both the *fusA* and the SOL-C assays delivered similar results and correctly identified 18 out of 21 *D. solani* strains but incorrectly identified two samples out of 54 non-*Dickeya solani* strains as *D. solani*. Based on *recA*

sequencing, the samples identified as *D. solani* using the assays were MK2, a *Duc3* strain from a new, slightly different clade to *D. solani* (Parkinson *et al.*, 2009) and 1121 (*D. zeae*), isolated from pineapple in 1962. Based on *recA* sequencing distinct lineages were identified and given the name DUC and further analysis to obtain additional characterisation data is required to better understand their relatedness to other *Dickeya* species within the *recA* complex (Parkinson *et al.*, 2009). Also based on *recA* sequencing, *D. zeae* was found to express the most genetic diversity and may account for the false positive obtained for 1121. A second evaluation using EUPHRESCO strains correctly identified all 11 *D. solani* strains but incorrectly identified PRI 3328 (*D. dianthicola*) as *D. solani.*

Due to the promising results from the two evaluations carried out at SASA and reported here, the assays were sent to six other institutes for further evaluation. Collaborative and comparative trials of diagnostic methods have been employed to ensure the rapid validation of new methods and are common in the development of diagnostic methods for human and animal pathogens (Chabirand *et al.*, 2014). So-called 'ring tests' have been recommended by the European and Mediterranean Plant Protection Organization (EPPO) to assess the specificity and sensitivity of new diagnostic methods. Ring tests help determine the variability of results from identical samples between laboratories and allow for an easy comparison of results. EN ISO 16140-1:2016 provides a protocol for the validation of alternative methods to ensure results obtained by new methods are, at a minimum, equivalent to those provided by the reference method and is applicable for the analysis of microorganisms in products intended for human consumption.

The primary reason for this ring test was to test the robustness and reproducibility of the real-time assays and serve as the first step in validation and acceptance of the methods by the EPPO. In total, 760 assays were carried out between six institutes using a variety of sample preparations and resulted in 13 strains incorrectly identified as *D. solani* and 168 *D. solani* not identified by the assays. It was decided that samples with a C_t value under 30 be considered positive for *Dickeya solani*, the threshold in the preliminary studies was a C_t value of under 25 indicating *D. solani*. If the assays using the lowest cell concentrations (10^3 CFU.ml^{-1}) were discounted, this would remove 127 of the false negative results, giving a total of 41 false negatives. It is clear from these results that the enrichment step had minimum effect on boosting the results which would imply that the concentration of cells was too low and could not be recovered sufficiently to facilitate an effective PCR reaction. It is also clear that boiling the cells seems to give a more

consistent set of results than extracting DNA prior to PCR. As DNA was not extracted by the same method in each laboratory, this approach cannot be universally discounted. A key issue with the laborious process of detecting *D. solani* is sample processing and DNA extraction as this remain the rate-limiting step in any of the test methods and continues to be a problem in this study (Mumford *et al.*, 2006).

A similar protocol was carried out by The EUPHRESCO FruitPhytoInterlab Group (2011) to assess detection methods for *Candidatus* Phytoplasma prunorum, *Candidatus* Phytoplasma mali and *Candidatus* Phytoplasma pyri. In this study, samples were sent in the form of DNA extracts to remove the requirement of sample manipulation. In the ring test for the *Dickeya solani* real-time assays a number of variables existed with only the assays themselves remaining consistent as the reagents were provided to the institutes alongside the samples. Samples had been sent by overnight courier to the individual institutes, after which time it was the responsibility of the institutes to culture and maintain the samples. It is possible that the samples were unable to survive long enough due to transit for the institutes to begin testing. There was also a lack of uniformity as to when the institutes started the method, which also affects the survival and ability to culture cells from the samples sent. Although the information was requested, the institutes did not return details of the cell numbers prior to beginning the experiment; therefore it is not possible to determine the exact inoculum levels prior to carrying out the assays. It is highly likely that the inoculum levels at the beginning of testing were either too low or the sample did not survive and therefore there were no cells to identify.

Two approaches were used to develop the real-time PCR assays specific to *Dickeya solani*. SOL-C was developed using genomic comparisons as described by Pritchard *et al.* (2013) and *fusA* was designed based on previous studies focussing on the housekeeping genes of *D. dianthicola* (Kowaleska *et al.*, 2010). Both sets of primers were validated against a wide range of *Dickeya* spp. and other enterobacteria and found to be specific for *D. solani*. The SOL-C primers have been further validated by NAK in the Netherlands and have been chosen in favour of other primers developed in the Netherlands. The SOL-C is currently being used at Fera for routine diagnostics (van der Wolf *et al.*, 2014). Both the *fusA* and SOL-C primers have been included in the list of preferred primers for testing within Europe through a EUPHRESCO *Dickeya* project to standardise testing throughout Europe (van der Wolf *et al.*, 2014). The approach using whole genome comparisons and *in silico* primer selection is a highly novel and good

potential for rapid diagnostics development for a range of future targets (Pritchard *et al.*, 2013).

Chapter 3. The Development of a Multilocus Sequence Analysis Typing System (MLSA) Specific for *Dickeya solani* and Identification of Sequence Differences using Pyrosequencing.

3.1 Introduction

3.1.1. *History of* Dickeya *sp.*

The genus *Erwinina* was established in 1920 to encompass plant pathogenic *Enterobacteriaceae,* and included *Erwinia carotovora* and *E. chrysanthemi* which were pectinolytic (Winslow *et al.,* 1920; Burkholder *et al.,* 1953). Due to the wide host range of *E. chrysanthemi*, the species was subdivided into six pathovars: *chrysanthemi*, *dianthicola*, *dieffenbachiae*, *paradisiaca*, *parthenii* and *zeae* on the basis of their host specificity, in addition to a biovar system based on key stable biochemical characteristics (Lelliott and Dickey, 1984; Samson *et al.,* 1987). The pectinolytic erwiniae were reclassified as member of the genus *Pectobacterium;* although, the name was not widely accepted until much later (Waldee, 1945; Hauben *et al.* 1998). Subsequent analysis of *Pectobacterium chrysanthemi* using 16S rDNA, DNA-DNA hybridisation and biochemical characterisation showed that it formed a unique clade distinct from *Pectobacterium* and formed a new genus: *Dickeya* (Samson *et al.,* 2005). Initially there were six species, which corresponded to the previous pathovar/biovar classifications within *E. chrysanthemi*: *D. dianthicola*, *D. dadantii*, *D. zeae*, *D. chrysanthemi* pv. *chrysanthemi*, *D. chrysanthemi* pv. *parthenii*, *D. paradisiaca* and *D. dieffenbachiae* (Samson *et al.,* 2005).

The first report of *Dickeya* sp. on potato in Europe was isolated in the Netherlands in the 1970s and prior to 2004, almost all *Dickeya* sp. were *D. dianthicola*. In 2005, new *Dickeya* strains were isolated which did not fall within any of the six species (Parkinson *et al.,* 2009; Sławiak *et al.,* 2009; Toth *et al.,* 2011). The development of genomic sequence analysis coupled with biochemical and REP-PCR analysis of strains of *Dickeya* sp. isolated from potato identified a new grouping within biovar 3, which was consistently isolated from seed potato tubers in a number of European countries and, at the time was given the unofficial name of "*Dickeya solani"* (Laurila *et al.,* 2008; Parkinson *et al.,* 2009) Subsequent detailed analysis of a wide range of isolates and reference strains from the genus *Dickeya* lead to the formal proposal of *Dickeya solani* (van der Wolf *et al*., 2014).

3.1.2 *Importance of Genetics in Plant Pathology*

For the effective control of the spread of plant pathogens, in addition to identifying the causative organisms, it is crucial to understand the ecology in order to control the disease. This can be achieved by understanding the diversity of the strains of the pathogens prevailing in each area. By understanding strain diversity, regulatory organisations are better able to detect and manage new disease threats. The development of genotyping methods such as DNA and ribosomal RNA (rRNA) sequencing methods and subsequent development of polymerase chain reaction (PCR) have facilitated phylogenetic classification and identification at various taxonomic levels (Sanger *et al.*, 1977; Woese and Fox, 1977; Saiki *et al*., 1988). By using the 16S rRNA gene, which occurs in all bacteria and archaea due to its role in protein synthesis and its subsequent evolutionary conservation, it has been possible to achieve fast identification of new isolates to already known taxa at a genus level, based on already published 16S rRNA gene sequences of type strains (Glaeser and Kampfer, 2015). Sequencing and phylogenetic analysis of 16S rDNA has been used to identify and differentiate between *Erwinia* spp., but, 16S rRNA does not generally allow for discrimination at a sub-species level and in order to achieve a higher phylogenetic resolution and discrimination within a species, performing additional phylogenetic analyses based on protein-coding genes, which are assumed to evolve at a slow but constant rate, provides better resolution at the genus level and below (Glaeser and Kampfer, 2015).

3.1.3 *Phylogenetic Studies Using Multiple Genes*

In general, analysis of a single protein-coding gene does not reflect general phylogenetic relationships. Multiple gene-based phylogenies which consider internal fragments of several protein-coding genes, referred to as multilocus sequence analysis (MLSA), allows the characterisation of closely related groups of strains (Gevers *et al*., 2005). MLSA is based on multilocus sequence typing (MLST) which was developed as a microbial typing method to study the epidemiology and genetics of pathogenic bacteria (Maiden *et al.*¸1998). MLST is based on the variation in housekeeping genes and internal gene fragments. Variations in the nucleotide sequences of the selected genes correspond to a unique allele of the gene, with each allele being assigned a number and the allele numbers from the genes included in the MLST are combined in a specific order and defined as the sequence type (ST) (Maiden, 2006). When strains share the same allelic profiles the strains are referred to as the same sequence type and

strains that share some of the alleles are still related but referred to as sequence complexes (Maiden, 2006).

The first MLST system studied the epidemiology of *Neisseria meningitides* and analysed 11 housekeeping genes (Maiden *et al.*, 1998). Further studies have suggested between 6-10 loci should be selected for MLST analysis, depending on the level of discrimination required (Maiden, 2006). Genes to be targeted include those related to pathogenicity and virulence as these are often linked to the evolution of strains and show greater diversity (Maiden, 2006). Over 50 individual MLST systems are now available and facilitate epidemiological studies through relationships between sequence types and sequence complexes and can make identification and classification of bacteria easier (Gevers *et al.*, 2005).

The terms MLST and MLSA are often used interchangeably, but both methods have distinctive uses and features. MLSA is useful for determining the relatedness of isolates by concatenating the sequences of gene fragments and using the concatenated sequences to determine phylogenetic relationships. Unlike MLST, MLSA assigns different levels of similarity based on the nucleotide differences; therefore, differences in the sequences of only a single nucleotide give a higher similarity than when multiple nucleotide differences are present. Consequently, MLSA is better suited for organisms that display clonal evolution rather than those in which recombinational events occur frequently and can also be applied to closely related species in order to determine phylogenetic relationships (Gevers *et al.*, 2005).

Phylogenetic trees created through the use of MLSA are more robust than phylogenies based on the evolution of a single gene and can derive a higher resolution power between species within a genus. Once a new strain has been identified to the genus level, MLSA is applied to determine the true relationship of the bacterial taxa and frequently it is necessary for the DNA fragments of several gene sequences to be concatenated to derive a deeper understanding of inter-relationships (Glaeser and Kampfer, 2015). For optimisation of MLSA it is necessary to select the most appropriate genes and typically housekeeping genes, which code for essential proteins with important functions are considered as they are stable in regards to rapid genetic modifications and their use makes it easier to extend MLSA studies (Gevers *et al.*, 2005).

3.1.4 *Pyrosequencing and Single Nucleotide Polymorphisms (SNPs)*

DNA sequencing has become one of the most important techniques in the study of biological systems and sequencing has been most commonly performed using dideoxy chain termination technology, typically Sanger sequencing (Sanger *et al.*, 1977). Although sequencing benefits from enhanced speed and sensitivity, there remain some limitations, especially in relation to cost and throughput and effort has been put in to establish alternative principles for sequencing. Alternatives have included sequencing by hybridization, parallel signature sequencing and pyrosequencing (Drmanac *et al*., 1989; Khrapko *et al*., 1989; Brenner *et al*., 2000; Ronaghi *et al*.,1996; Ronaghi *et al*., 1998). Pyrosequencing in particular has been shown to be successful for both confirmatory and *de novo* sequencing although it has not been applied to whole genome sequencing because of its limitation in the length of sequence that can be read (Ronaghi, 2001).

Pyrosequencing was developed in 1996 by Ronaghi *et al.* and is considered "sequencing by synthesis," that is, it relies on the detection of pyrophosphate release during nucleotide incorporation rather than chain termination with dideoxynucleotides as with Sanger sequencing. Sanger sequencing allows for DNA sequencing of lengths between 800-1000 base pairs, whilst pyrosequencing is limited to fragment lengths of 300-400 nucleotides. Pyrosequencing has been found to be helpful for applications such as genotyping, resequencing of virulence genes and sequence determination of difficult secondary DNA structure (Ronaghi *et al.*, 1999). Pyrosequencing is based on the detection of released pyrophosphate (PPi) during DNA synthesis and through a cascade of enzymatic reactions, visible light is generated which is proportional to the number of incorporated nucleotides. The desired DNA sequence is determined by the light emitted when the next complementary nucleotide is incorporated. Only one of the four possible nucleotides (A, T, C, or G) is added and available at a time so only one nucleotide is incorporated on the single stranded template and the intensity of the light determines if there are more than one of the nucleotides in a row. If the added nucleotide is not incorporated, then it is degraded before the next nucleotide is added for synthesis. This process is repeated with each of the four nucleotides until the DNA sequence is determined (Fakruddin *et al.*, 2012).

Pyrosequencing has been successfully used to genotype and understand environmental biological diversity and in diagnosing clinical bacteria (Weinstock, 2012; Roh *et al.*,

2010; Fonseca *et al.*, 2010). This technique has the potential to complement, and in some cases replace, culture-based, biochemical and immunological assays in microbiology studies and allows for the identification of species which it may not be possible to culture (Weile and Knabbe, 2009; Roesch *et al.*, 2007; Quince *et al.*, 2009). Predicting and ranking potential pathogens remains a challenge in biosecurity and current detection and inspection methods are laborious and time-consuming (Paini *et al.*, 2010). Pyrosequencing provides real-time data without the need for gels, probes or labels and allows for the characterization of single nucleotide polymorphisms (SNPs) in addition to insertion-deletions and unknown sequence variants and can sensitively quantify allele frequencies and DNA methylation levels (Koontz *et al.*, 2009). Pyrosequencing has become the fastest method for sequencing PCR products and generates an accurate quantification of mutated nucleotides (Ronaghi, 2001). It allows for the efficient analysis of large amounts sequences whether from a single genome, a single gene or a community of micro-organisms and can help identify microbial strains responsible for outbreaks of disease, tracking evolutionary history and disease spread and developing new diagnostic tests based on PCR technologies (Relman, 2011). Single nucleotide polymorphisms are variations in a single nucleotide that occurs at a specific position in the genome (Gharizadeh *et al.*, 2007). SNPs can occur in the coding and non-coding regions of genes or in the intergenic spacer regions; however, it should be recognized that when SNPs occur in a coding region it does not necessarily change the amino acid sequence of the protein that is produced (Barreiro *et al.*, 2008).

Pyrosequencing has been applied to plant pathogenic pathogens and has provided characteristic sequence information of the amplified phytopathogens to discriminate strains of *Xanthomonas* and serves as the starting point for the application and development of pyrosequencing in plant inspection and quarantine (Gan *et al.*, 2014). A study using *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye, a pathogen with wide geographic distribution, has demonstrated that pyrosequencing has the potential to be to be applied to plant inspections and quarantine which are important in agriculture (Gan *et al.*, 2014).

Studies using pyrosequencing of *Xanthomonas* show that there are major advantages to using pyrosequencing in the detection of pathogens including a high detection limit, and including an extra step for the concentration of bacterial cells can improve the detection sensitivity (Gan *et al.*, 2014; Gehring *et al.*¸ 2012). Pyrosequencing also offers time saving as complete detection can be carried out in two hours, with the results being

achieved in real-time and a number of samples can be analysed simultaneously (Gan *et al.*, 2014). Studies have shown that pyrosequencing is an accurate, rapid, sensitive and efficient detection method for plant pathogens (Gan *et al.*, 2014).

MLST and Pyrosequencing sequencing have been used successfully in the study of a particular strain of *Xylella fastidiosa*, a xylem-inhabiting bacterium with slow *in vitro* growth, which is considered a quarantine organism in the European Union (Montes-Borrego *et al.*, 2015). The pathogen has been recently associated with quick decline syndrome in olives (OQDS) in Italy and has quickly expanded to a wider area (Montes-Borrego *et al*., 2015). Preliminary phylogenetic analysis based on *gyrB*, the gene encoding for the β-subunit polypeptide of DNA gyrase, indicated that the isolates from the olive trees are closely related to the branch associated with the subspecies *pauca* which was confirmed by MLST, which indicated it was a distinct strain within the subspecies *pauca* (Loconsole *et al.*, 2014).

3.1.5 *Specific Aims of Developing a MLSA System for* Dickeya solani.

One aim of this study was to develop a MLSA system for *Dickeya* sp. based on previous work focusing on *D. dianthicola*. In the initial study carried out on *D. dianthicola* by Kowalewska *et al.*, (2010), seven housekeeping genes were targeted which has been extended in this work to include two additional genes. This system will allow direct comparisons to be made with strains characterised in Great Britain to those from other parts of the world. Sequencing based MLSA analysis lends itself to the construction of an online database which other researchers can access to help them characterise new strains world-wide. In addition, *D. solani* characterisation was carried out to a higher level of resolution through the identification of possible SNPs by utilising Pyrosequencing technology.

3.2. Materials and Methods

3.2.1 *Producing Preliminary PCR Product for MLSA*

The multilocus sequence analysis (MLSA) system specific for *Dickeya solani* was based on a previously designed method for *Dickeya dianthicola* (Kowalewska *et al*., 2010). The original system focused on seven housekeeping genes; which were included in this study; in addition to two more genes being assessed: *dnaJ* and *dnaX*, as they had been found to be particularly effective at separating other members of the family *Enterobacteriaceae* in previous studies *(*Parkinson *et al.* 2014; Sławiak *et al*., 2009).

Reaction mixtures are described in Tables 3.3 and 3.5, using the external primers indicated in Table 3.1. To these, 1µl of DNA (extracted as described in Appendix 2) of stock isolates (list found in Appendix 1) was added. Temperature cycling was carried out using Veriti machines and described in Tables 3.4, 3.6 and 3.7. The resultant PCR products were purified by adding 0.5µl each of shrimp alkaline phosphate (SAP) and exonuclease (EXO) and incubated at 37^{o}C for 45 minutes, 80^{o}C for 15 minutes. Products were stored at 4^{o}C before sequencing.

Table 3.1 External Primers Used for the Initial PCR Reaction as Part of the MLSA Analysis

Gene	Forward primer	Reverse primer
*recA**	5'-GGTAAAGGGTCTATCATGCG-3'	5'-CCTTCACCATACATAATTTGGA-3'
*dnaN**	5'-GGTACCGATCTGCAAATGGAGA-3'	5'-TCTTCCTGTTCCGGGTTGTT-3'
*fusA**	5'-CACCGGTGTGAACCACAAAA-3'	5'-TAGCCTTTCGGATTTGAGCC-3'
*gapA**	5'-AAGTGAAAGACGGTCACCTGGT-3'	5'-CGATCAGGTCCAGAACCTTGTT-3'
*infB**	5'-AAATCACTGGCCGCAGAGAT-3'	5'-TGACCTGAGAACCTTTAACAGCC-3'
*purA**	5'-AGAACGTTGTCGTACTGGGCA-3'	5'-AGTGGAGTAGGCTTTGACGATACC-3'
*rplB**	5'-TTAACCCTGAGCTGCACAAGG-3'	5'-GCGGCGTACGATGAATTTATC-3'
dnaJ	5'-AA**R**AA**R**GC**K**TA**Y**AA**R**CG**K**CTGGCGATGAA-3'	5'-CGGATCTCTTT**V**GTGACGCC**R**CG-3'
dnaX	5'-TCGACATCCA**R**CGCYTTGAGATG-3'	5'- TATCAGGT**Y**CTTGCCCGTAAGTGG-3'

*designed by Kowalewska *et al*., 2010

dnaJ designed by Parkinson *et al.*, 2014.

dnaX from Sławiak *et al*., 2009

Letters K, R, V and Y represent a position where more than one nucleotide could occur:

K – G or T

R – G or A

V – G, C or A

Y – T or C

Table 3.2 Internal Primers Used for Sequencing Genes as Part of the MLSA Analysis

Gene	Forward primer	Reverse primer
*recA**	5'-GGTAAAGGGTCTATCATGCG-3'	5'-CCTTCACCATACATAATTTGGA-3'
*dnaN**	5'-GGCCCGTAAGCTGTTCGATAT-3'	5'-ACCGGCCATCCACCAGTTT-3'
*fusA**	5'-GTTTATTGTGCGGTAGGTGGTG-3'	5'-CTTGAACGCAGAACCACAGGTA-3'
*gapA**	5'-ACGCAAACACATTCAGGCTG-3'	5'-TTTCACGAAGGTGTCGCTCA-3'
*infB**	5'-TGCATCGGACTCTGTGACTCA-3'	5'-TTTTCTTCTGCCATCCGGC-3'
*purA**	5'-GGCCAAATATGTTGTGCGCT-3'	5'-CCGTTTTGTAGTAATGCACCAGC-3'
*rplB**	5'-GTTATATCCTGGCGCCGAAA-3'	5'-GCGGGTCTTCTTACCTTTGGT-3'
dnaJ	5'-AA**R**AA**R**GC**K**TA**Y**AA**R**CG**K**CTGGCGATGAA-3'	5'-CGGATCTCTTT**V**GTGACGCC**R**CG-3'
dnaX	5'-TCGACATCCA**R**CGC**Y**TTGAGATG-3'	5'- TATCAGGT**Y**CTTGCCCGTAAGTGG-3'

*designed by Kowalewska *et al*., 2010

dnaJ designed by Parkinson *et al.*, 2014

dnaX from Sławiak *et al*., 2009

Letters K, R, V and Y represent a position where more than one nucleotide could occur:

K – G or T

R – G or A

V – G, C or A

Y – T or C

Table 3.3 Conventional PCR Reaction Mix (except *dnaJ*)

Component	Volume per reaction (µl)
Sigma Jumpstart ReadyMix	10
Forward primer (10pmol/µl)	1
Reverse primer (10pmol/ µl)	1
Sigma water	7
DNA	1
Final volume	**20**

Table 3.4 PCR Cycles (except *dnaJ* and *dnaX*)

Temperature	Time	
94˚C	30 s	x 35 cycles
94˚C	30 s	
60˚C	30 s	
72˚C	1 min	
72˚C	7 min	
4˚C	∞	

Table 3.5 *dnaJ* Conventional PCR Reaction Mix

Component	Volume per reaction (µl)
Sigma Jumpstart ReadyMix	12.5
Forward primer (10pmol/ µl)	0.5
Reverse primer (10pmol/ µl)	0.5
Sigma water	10.5
DNA	1
Final volume	**25**

Table 3.6 *dnaJ* PCR Cycles

Temperature	Time	
95˚C	5 min	x 40 cycles
94˚C	30 s	
60˚C	1 min	
72˚C	1 min	
72˚C	7 min	
4˚C	∞	

Table 3.7 *dnaX* PCR Cycles

Temperature	Time	
95˚C	5 min	x 35 cycles
94˚C	1 min	
59˚C	1 min	
72˚C	2 min	
72˚C	5 min	
4˚C	∞	

After clean-up of the PCR product with EXO-SAP, 1µl was added to the reaction mix found in Table 3.8, using the internal primers from Table 3.2. Reactions were carried out in duplicate and under the BigDye PCR conditions indicated in Table 3.9.

Table 3.8 Reaction Mix for Sequencing

Component	Volume/reaction (µl)
ABI Big Dye 3.1 reaction mix	0.5
ABI Big Dye 5x buffer	1.75
Primer 10 µM (Forward OR reverse)	0.5
RNase Free Water	6.25
PCR product to be added	1
Final Volume	**10**

Table 3.9 Big Dye V3.1 Sequencing Programme

Temperature	Time	
96˚C	1 min	x 25 cycles
96˚C	10 s	
50˚C	5 s	
60˚C	4 min	
4˚C	∞	

3.2.2 *Sequencing of Internal Fragments*

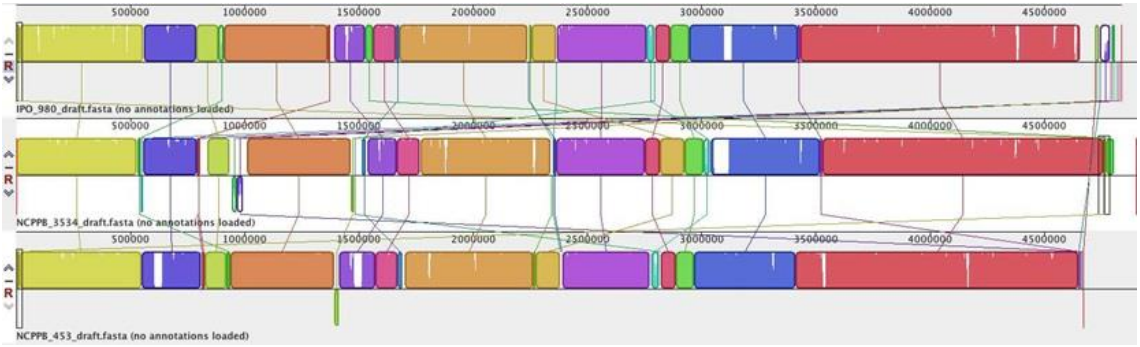
To each resultant PCR reaction, 1 µl of 3 M sodium acetate (pH5.2), 2 µl dH_2O and 25 µl 96% ethanol (aq., v/v) was added. The PCR plate was agitated at 1,400 rpm for 30 seconds. The samples were incubated in the dark for 15 minutes and then centrifuged at 3,000*g* for 35 minutes. Ethanol was removed from the samples by inverting the plate, placing it on paper towel in the centrifuge rotor and then pulse spinning up to 185*g*. A further 50 µl of 70% ethanol (aq., v/v) was added to each sample well and centrifuged again at 3,000*g* at 4ºC for 15 minutes. Ethanol was removed as described previously. Wells were dried by placing in the PCR machine and increasing the block temperature to 95ºC. To each reaction well, 10 µl of Hi-Di formamide (Applied Biosystems) was added and then incubated at 95ºC for 5 minutes. The plate was snap cooled using a cold block, which had been stored at 4ºC, for 1 minute and the plate was spun briefly before placing in the sequencer. The sequencer used was ABI 3130XL Genetic Analyzer and analysis carried out using SeqMan Pro (DNASTAR Lasergene 11). The results were analysed using 'Sequencing Analysis' software (Applied Biosystems Inc; Foster City, CA, USA). Using Lasergene-SeqMan Pro 11 (*DNASTAR* Inc., Madison, WI, USA) the forward and reverse sequences were assembled and refined by eye to determine the consensus sequence which was stored as a contig file. Contig files were copied into MEGA5 software (Centre for Evolutionary Functional Genomics, AZ, USA) and maximum likelihood trees for each gene were constructed using single linkage clustering and the 'tree function'. The bootstrap percentages were calculated within 500 replicates.

3.2.3 *Identification of Potential Differences Between* Dickeya solani *Genomes Using Pyrosequencing*

The genomes of three *Dickeya solani* strains, MK10, MK16 and IPO2222, each isolated from different environmental samples from various geographical locations were sequenced at the Centre for Genomics Research at Liverpool University (Pritchard *et al.*, 2013). MK10 was originally isolated in 2006 in Scotland from a rotten tuber recovered from a shipment of ware potatoes from Israel, MK16 was isolated from a Scottish river in 2008 and IPO2222 was isolated from a tuber in the Netherlands in 2007. With this sequence information, individual, putative Single Nucleotide Polymorphisms (SNP) markers were identified using the Mauve Multiple Genomic Alignment system (Darling *et al.*, 2010).

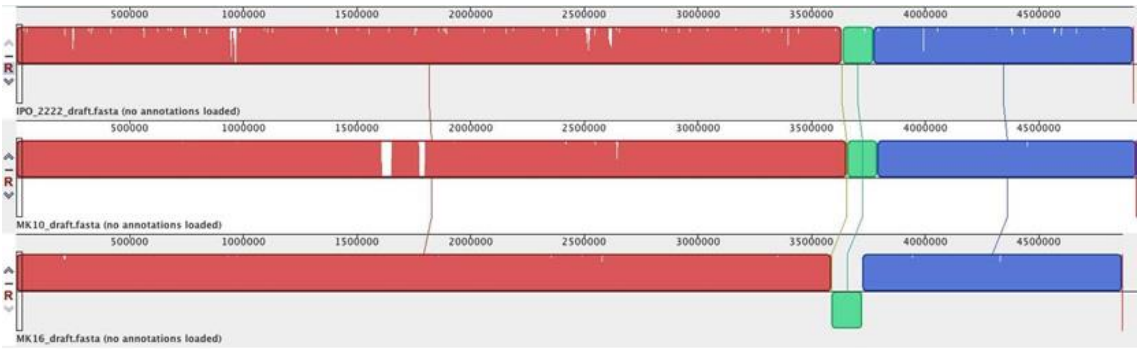
The three *Dickeya solani* sequences were supplied along with 13 other *Dickeya* spp. genomes by Leighton Pritchard and Ian Toth from JHI, Dundee. The 16 genomes, in the form of Mauve and RAST annotations, were stitched together and the junction points were identified by linker regions. The sequences were aligned using the Mauve system. Figure 3.1. shows the *D. dianthicola* alignments and Figure 3.2 shows the *D. solani* alignments. The different colour blocks used in the figures represent regions of genomic alignment. These regions are largely homologous and internally free from genomic rearrangement. Blocks above the line are orientated relative to the first genome and those below are in the inverse orientation. Non-coloured regions denote sections that lack homology with the other genomes analysed. Blocks of the same colour are connected by lines between each of the genomes and indicate the extent of regions of high homology. The limits of each block are determined by areas of genomic rearrangement, or regions where there has been a sequence loss or gain. In Figure 3.1, it is clear that *D. dianthicola* contains more sequence variation than *D. solani* which is more conserved, as indicated in Figure 3.2. Using the Mauve SNP calling function, SNP markers were identified and manually examined. SNPs containing 'N', used to indicate any base could be present at this location, were ignored and determined to be junctions. SNPs containing gaps were also rejected and were found to be missing data from junctions. The sequences that remained were considered to be potential locations for SNPs. From the initial analysis, the three *D. solani* genomes were found to have 187 potential SNPs; in contrast to the three *D. dianthicola* genomes included in this study which were found to contain 30,526 SNPs.

Figure 3.1 Mauve Alignments of *Dickeya dianthicola* Genomes



The order of *Dickeya dianthicola* isolates, from top to bottom: IPO980, NCPPB3534, NCPPB453

Figure 3.2 Mauve Alignments of *Dickeya solani* Genomes



The order of *Dickeya solani* isolates, from top to bottom: IPO2222, MK10, MK16.

The first analysis of *Dickeya solani* SNPs showed clustering of SNPs within the genomes. For example, IPO2222 contained 36 SNPs in a space of approximately 200 base pairs, between positions 945976 AND 946177. Assuming closely related SNPs indicated an issue such as a mis-assembly of contigs then the number of SNPs fell considerably from the original 187 SNPs to 23 SNP markers by discarding co-located SNPs.

3.2.4 *Confirmation of Potential SNPs*

In total, twenty-one potential SNP markers were identified. Due to the identification of the SNPs thus far being based on the sequence alignments from JHI, to confirm their presence, SASA isolates of MK10, MK16 and IPO2222 were sequenced, focusing on

the locations of the potential SNPs. The number assigned to the SNP refers to the approximate location of the SNP on the genome. Primers were designed using the original three complete *D. solani* genomes provided by JHI and using Mauve Multiple Genomic Alignment (Darling *et al*., 2010). Sequencing was carried out using the method described in Chapter 2, using the Reaction Mix and PCR cycles as described below in Tables 3.10 and 3.11. The sequence regions were compared and eight SNPs were identified within the three isolates from the SASA collection. Focussing on these 8 SNPs, a further eleven *D. solani* isolates were analysed to determine whether the SNPs were present in other *D. solani* strains.

Table 3.10 SNP Sequencing PCR Reaction Mix

Component	Volume per reaction (µl)
Sigma Jumpstart ReadyMix	12.5
Forward primer (10pmol/ µl)	0.5
Reversed primer (10pmol/ µl)	0.5
Sigma water	9.5
DNA	2
Final volume	**25**

Table 3.11 SNP Sequencing PCR Cycles

Temperature	Time	
95˚C	15 min	x 45 cycles
94˚C	30 s	
60˚C	30 s	
72˚C	30	
72˚C	10 min	
4˚C	∞	

3.2.5 *Pyrosequencing Assay Development*

Pyrosequencing primers, outlined in Table 3.13 were designed using the Pyromark Assay Design 2.0 (Qiagen) and synthesised by Eurofins. Either the forward or reverse primer was labelled with biotin and is highlighted in bold in Table 3.13. When designing the primers the following conditions were considered: the ideal length of the primer would be between 18 and 24 bases with an approximate equal distribution of GC and AT, ideally more GC rich in the 5' end. The sequencing primer would be positioned within 5 bases of the SNP, but ideally only one base would be between the primer and SNP of interest. The T_m would fall within the range of 62-68^{o}C, and PCR cycle would result in an optimal PCR amplicon of between 80 and 200 base pairs. Once designed, primers were made to a concentration of 100µM and stored at a working concentration of 10µM.

To acquire the PCR products for the Pyrosequencing reaction, an initial conventional PCR reaction was carried out on a Veriti® Thermal Cycler (Life Technologies). The reaction mix is described in Table 3.10 and with the addition of the DNA sample, gave a final volume of 25µl. The PCR cycles are indicated in Table 3.11. PCR products were confirmed by visualisation using gel electrophoresis as described in Appendix 2 and stored at either 4^{o}C overnight or at -20^{o}C for longer term storage.

Immobilization of the PCR product was carried out by attaching the PCR product to Sepharose beads. For each PCR product, the reaction mix outlined in Table 3.12 was added to 10 µl of PCR product. This was achieved by dispensing 70 µl of master mix into a well of a 96-well PCR plate (VWR) and then adding 10 µl of PCR product. The wells were sealed with strip caps (Ranin) and agitated at 1400 rpm for 5-10 minutes at room temperature on a MixMate PCR 96 (Eppendorf).

Table 3.12 Reaction Mix for Immobilization of PCR Products

Component	Volume per reaction (µl)
Binding Buffer (Qiagen)	40
Streptavidin Sepharose High Performance Beads* (GE Healthcare)	2
Sigma Water	28

The sequencing primers indicated in Table 3.13 were diluted to 0.3µM with PyroMark Annealing Buffer (Qiagen) and SNP reactions were performed on a Pyromark Q24 (Qiagen) following the manufacturer's protocols (Pyromark Q24 user manual, Qiagen). The runs were analysed with the PyroMark Q24 Software (Qiagen).

Table 3.13 SNPs Pyrosequencing Primers

SNP	Forward 5'-3'	Reverse 5'-3'	Sequencing Primer 5'-3'
117	AACGGTTAGGCTTTGTCCTGT	CGCTCGTCAGTACTCCGATTTT*	GGTTAGGCTTTGTCCTGT
118	CAGCATGAAGTGCGTATCCG*	TGCTGCAACAGGTGATAGGC	GCAACAGGTGATAGGCT
172	TTCCGCTTCAATGGCATGTC*	TGAACGTGAATCCGGACTGC	TGAATCCGGACTGCG
311	AACATTCCGAGAGGTTTGAGTTTA*	CCTTTTATTGCTTACCGGAAACTG	TTACCGGAAACTGTCA
446	CGCCATTGTCCTCGTTTATCTCTA*	AGGAAATTTCAGGCGGCATG	TTATTGACTTCTTTTTCTCG
523	GTCGGTCACGCGCGAATA	ACCCGCTGAGTACCCCTCC*	CGGTAATGCGGTAGG
799	CTACTGAAAACCTCCCTCTTCAAA	TGTGCGCCATCAGACATAA*	CCCTCTTCAAAATGGA
834	TTCATCGGCAATCACGGT	ACGCCTGCTGATCCTCGA*	TCTCCGCTTTCCACT

*Biotinylated primer

3.3. Results

3.3.1 *Results from MLSA Analysis*

The relatedness and phylogenetic relationships of isolates analysed with MLSA is determined by concatenating the sequences of gene fragments of the housekeeping genes. A higher similarity is given to isolates which differ by only a single nucleotide and a lower similarity is awarded between isolates with multiple nucleotide differences. This makes MLSA suitable to organisms assumed to have a clonal evolution.

In a comprehensive study of 37 representatives of the *Dickeya* taxa, 17 of which were *Dickeya solani*, a multilocus sequence analysis (MLSA) approach was used to determine diversity between *D. solani* isolates and also between *Dickeya* species and related genera. In total, nine housekeeping genes were employed, seven of which were used previously in a study focused on *D. dianthicola* diversity (Kowalewska *et al*., 2010) and two additional genes: *dnaX* and *dnaJ*.

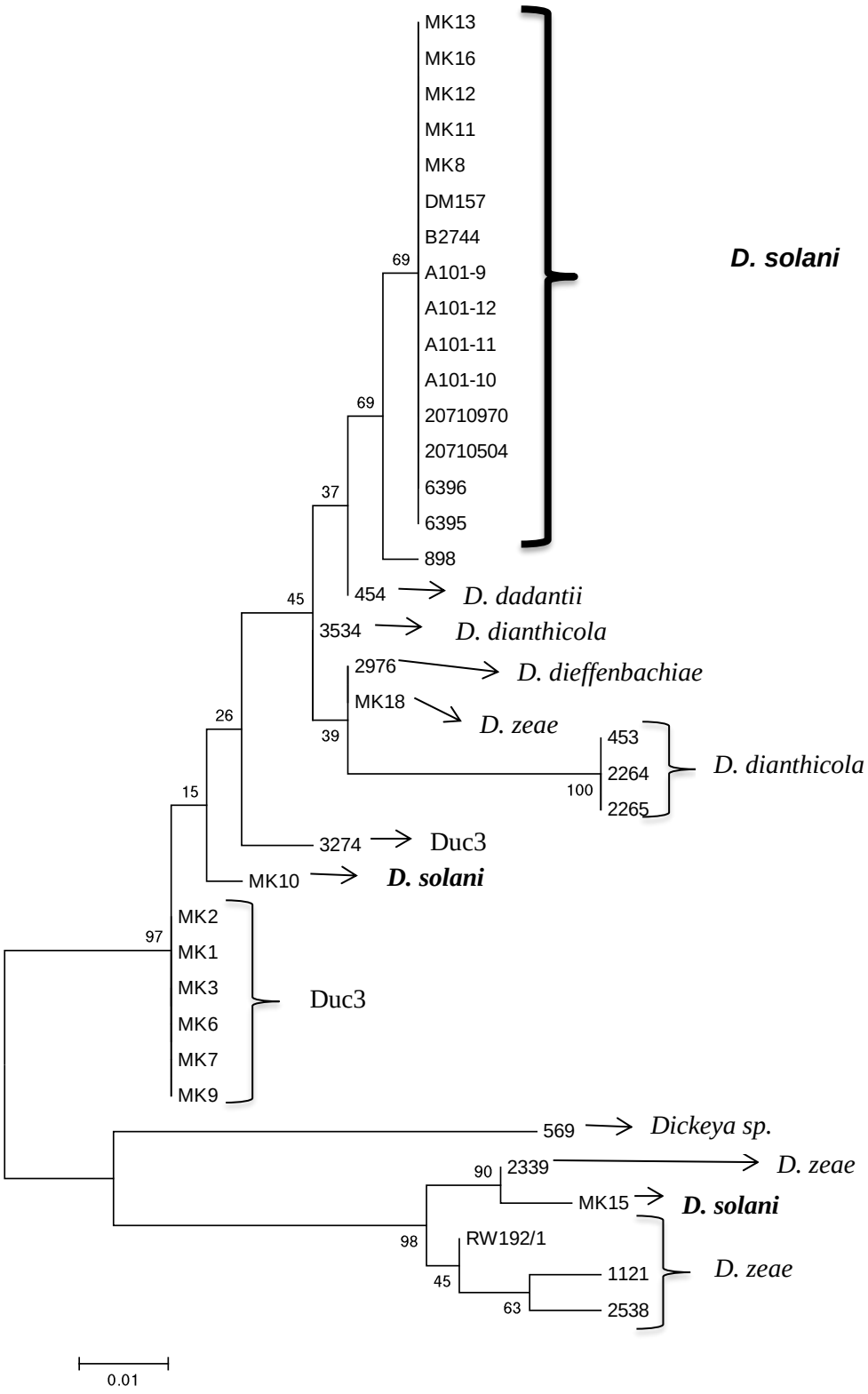


Figure 3.3. Maximum Likelihood Tree of *dnaJ* Sequence Data

In total, 37 strains were analysed using the *dnaJ* gene, 17 of which were *Dickeya solani* and constructed into Figure 3.3 phylogenetic tree The only homologous clade formed was that of 15 *D. solani* strains with other *Dickeya* species, including *D. dianthicola*, displaying differences between the species isolates. MK10 and MK15, both *D. solani*, do not fall within the *D. solani* clade. MK10 was more closely related to *Dickeya* sp. identified as Duc3 (Parkinson *et al.*, 2009). MK15 was found to be more similar to *D. zeae*, based on the *dnaJ* gene. Variation between the other *Dickeya* spp. strains was also seen with no other species forming homologous clades.

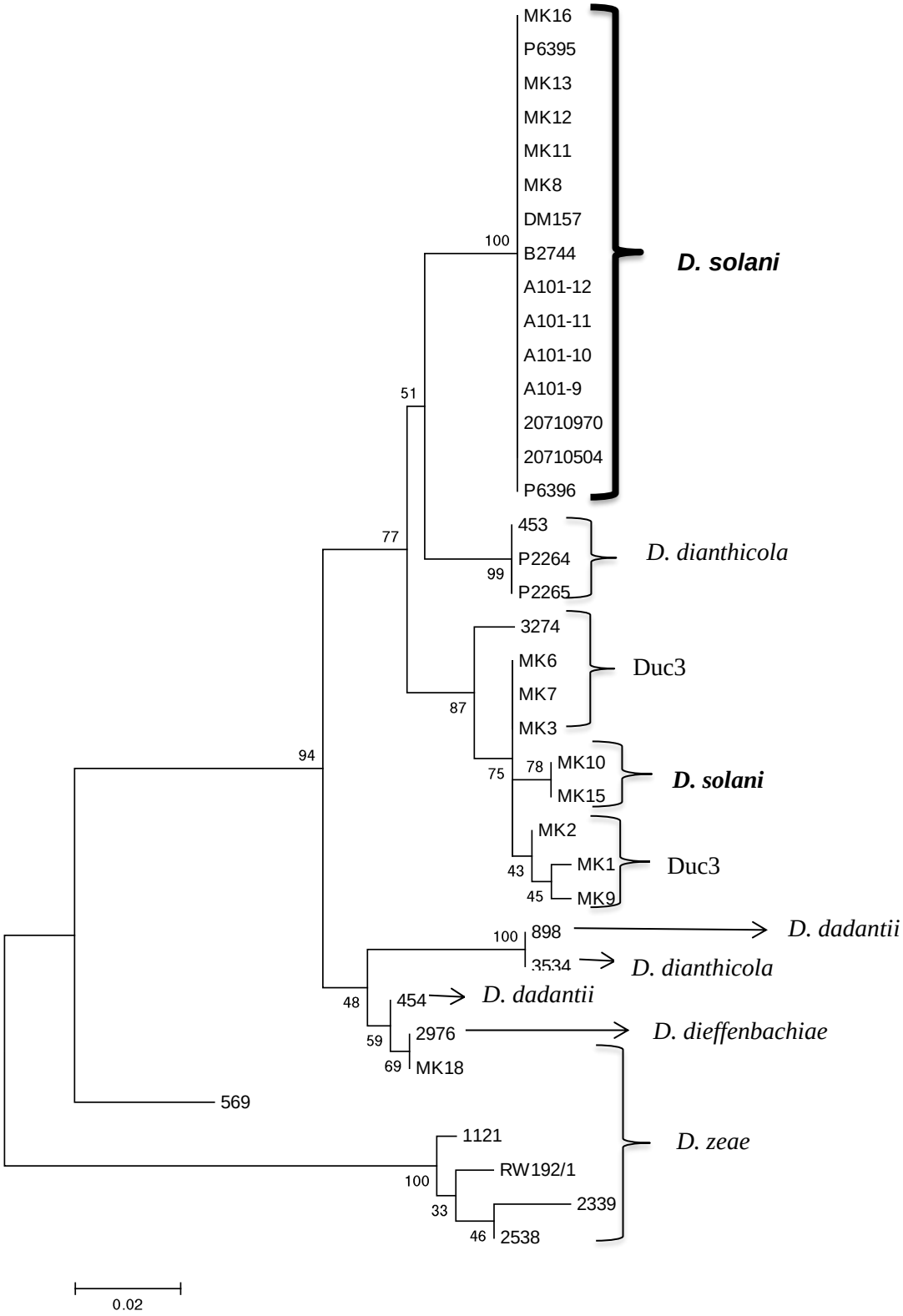


Figure 3.4. Maximum Likelihood Tree of *dnaX* Sequence Data

A total of 36 isolates were analysed, 17 of which were *D. solani*, using the *dnaX* gene and once again, *D. solani* formed a homologous clade, as indicated in Figure 3.4. As with *dnaJ*, MK10 and MK15 did not fall within the *D. solani* grouping. Based on their *dnaX* sequence, MK10 and MK15 showed no differences and grouped between Duc3 isolates (as defined by Parkinson *et al.*, 2009).

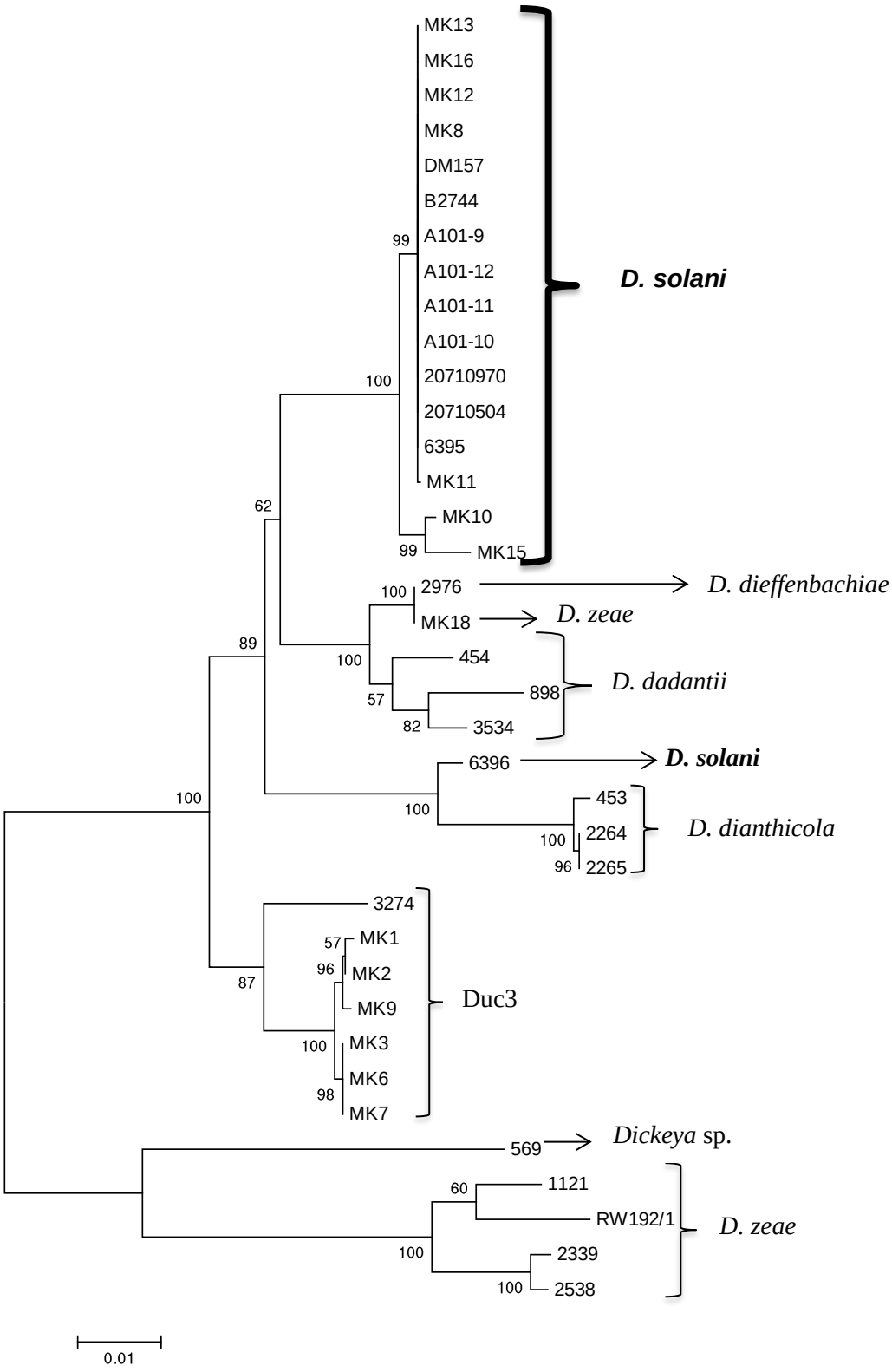


Figure 3.5 Maximum Likelihood Tree of Concatenated Sequence Data Using All Nine Housekeeping Genes

Using the concatenated sequences of the nine housekeeping genes, *D. solani* is grouped into a homologous clade of 14 out of the 17 *D. solani* isolates, with MK10 and MK15 expressing slight differences but being most similar to the other *D. solani* isolates, as indicated in Figure 3.5. Isolate P6396 showed differences to the *D. solani* strains, despite its identification as *D. solani* based on *recA* sequencing and falling into the homologous clade based on *dnaJ* and *dnaX* and based on the real-time assays.

The other *Dickeya* isolates mostly group based on their species identification with only MK18 and 569 not grouping as expected. MK18 has been identified as *D. zeae* based on *recA* sequencing; but does not show any similarity with the other *D. zeae* isolates based on *dnaX, dnaJ* and the concatenated analysis. In all three analyses MK18 is shown to be identical to 2976 which was identified as *D. dieffenbachiae*; consequently it is possible that MK18 was originally identified incorrectly as *D. zeae* and is in fact *D. dieffenbachiae*.

Based on the MLSA data, little variation was seen amongst the *D. solani* isolates, with only three out of the 17 isolates used showing any differences. There were no sequence differences between 13 out of the 17 *D. solani* isolates used for any of the genes *dnaJ, dnaX* and for the concatenated data, which suggests that *D. solani* is a clonal pathogen.

3.3.2 *Results of Identification of SNPs and Pyrosequencing Studies*

Eight potential SNPs were identified to characterise strains of *Dickeya solani*, and named according to the location of the SNP in the alignments of three *D. solani* strains which had been sequenced at the James Hutton Institute and indicated in Figure 3.2. Six unique SNP profiles of the eight SNPs were found from the 14 *D. solani* isolates analysed and are presented in Table 3.14. The 14 *D. solani* isolates used formed part of the collection used in the EUPHRESCO project, are described in Table 3.15 with the sequence variations of the SNPs indicated in Table 3.16.

Group 1 refers to the largest grouping and is composed of MK11, MK14, MK15, MK16, A101-9, A101-10 and A101-11. The isolates in Group 1 come from an Israeli ware import (MK11), three strains from an infested river in South-East Scotland (MK14, MK15 and MK16) and isolates from Polish Potatoes (A101/9, A101/10 and A101/11). The second largest group is referred to as Group 2 and contains three isolates: B2745, DM157 and DM159. The isolates come from a Belgian variety which was undergoing trials at SASA (B2745) and two isolates from an infected crop of cv. Agria grown in Scotland and had been produced from Dutch-origin seed which had

been grown once in England (DM157 and DM159). The other strains: IPO2222, MK10, B1 and B2744 produced unique profiles and were recovered from Dutch potato (IPO2222), an Israeli ware import (MK10), a Spanish ware import (B1) and a Dutch variety undergoing trialling at SASA (B2744). IPO2222 is considered the type strain for *Dickeya solani*.

The sequences identified including each SNP, with the SNP indicated in bold and underlined, and the strains associated with the sequences are described below:

SNP 117

All strains analysed had the sequence GTG**G**GCCTT with the exception of MK10 which replaces one of the guanine with adenine, giving a sequence of GTG**A**GCCTT

SNP 118

All strains expressed the SNP 118 sequence ATG**A**AAG, whilst in IPO2222 and MK10 the adenine was replaced with a cytosine to give the sequence ATG**C**AAG.

SNP 172

Twelve of the strains analysed had the sequence C**A**CGCAGT for the SNP 172; however in B1 and MK10, an adenine was replaced with a guanine.

SNP 311

For SNP 311, Group 1 strains, B2744 and B1 displayed the sequence GATATT**A**TCT. Those strains in Group 2, IPO222 and MK10, an adenosine was replaced with a guanine, giving the sequence GATATT**G**TCT.

SNP 446

Group 1 strains, B1 and MK10 had a sequence of GGA**G**CGAG, and for group 2 strains, IPO2222 and B2744 the sequence contained an adenosine instead of guanine: GGA**A**CGAG.

SNP 523

All strains when analysed for SNP 523 had the sequence GGG**A**CCG with the exception of B1 and MK10 which replaced one of the adenosines with a guanine: GGG**G**CCG.

SNP 799

Group 1 strains, B1 and B2744 had the sequence AAATGGAAG**T**CTA when analysed for the SNP 799. Group 2 strains, IPO2222 and MK10 replaced a thymine with cytosine to give the sequence AAATGGAAG**C**CTA.

SNP 834

All strains analysed, with the exception of MK10, had the sequence GCCGGATTT**G**C when analysed for SNP 834. In MK10, a guanine is replaced with an adenosine to give the sequence GCCGGATTT**A**C.

Table 3.14 Presence of Individual SNPs in *Dickeya solani* Isolates Identified Using Pyrosequencing

Strains/SNPs	117		118		172		311		446		523		799		834	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
MK16		X		X		X		X		X		X		X		X
MK11		X		X		X		X		X		X		X		X
MK14		X		X		X		X		X		X		X		X
MK15		X		X		X		X		X		X		X		X
A101-9		X		X		X		X		X		X		X		X
A101-10		X		X		X		X		X		X		X		X
A101-11		X		X		X		X		X		X		X		X
B2745		X		X		X	X		X			X	X			X
DM157		X		X		X	X		X			X	X			X
DM159		X		X		X	X		X			X	X			X
IPO2222		X	X			X	X		X			X	X			X
B1		X		X	X			X		X	X			X		X
B2744		X		X		X		X	X			X		X		X
MK10	X		X		X		X			X	X		X		X	

X indicates SNP present

Group 1 strains	
Group 2 strains	

Table 3.15 Strains Used in Study of SNPs (from EUPHRESCO project)

Strain	Attributed species	Host	Country	Date of Isolation
MK10	*Dickeya solani*	*Solanum tuberosum*, rotten tuber.	Israel	05/04/06
MK11	*Dickeya solani*	*Solanum tuberosum*, asymptomatic tuber	Import	27/04/06
MK14	*Dickeya solani*	River 2	Scotland	21/07/08
MK15	*Dickeya solani*	River 2	Scotland	30/07/08
MK16	*Dickeya solani*	River 2	Scotland	30/07/08
A101/9	*Dickeya solani*	*Solanum tuberosum*	Poland	
A101/10	*Dickeya solani*	*Solanum tuberosum*	Poland	
A101/11	*Dickeya solani*	*Solanum tuberosum*	Poland	
B2744	*Dickeya solani*	*Solanum tuberosum*	Scotland	2009
B2745	*Dickeya solani*	*Solanum tuberosum*	Scotland	2009
B1	*Dickeya solani*	*Solanum tuberosum* tubers	Spain	2009
DM157	*Dickeya solani*	*Solanum tuberosum* cv. Agria	Scotland (Dutch seed)	2009
DM159	*Dickeya solani*	*Solanum tuberosum* cv. Agria	Scotland (Dutch seed)	2009
IPO2222	*Dickeya solani*	*Solanum tuberosum*	Netherlands	2007

Table 3.16 Identification of Differences of Individual SNPs

SNP	Sequence
117a	GTG**A**GCCTT
117b	GTG**G**GCCTT
118a	ATG**C**AAG
118b	ATG**A**AAG
172a	C**G**CGCAGT
172b	C**A**CGCAGT
311a	GATATT**G**TCT
311b	GATATT**A**TCT
446a	GGA**A**CGAG
446b	GGA**G**CGAG
523a	GGG**G**CCG
523b	GGG**A**CCG
799a	AAATGGAAG**C**CTA
799b	AAATGGAAG**T**CTA
834a	GCCGGATTT**A**C
834b	GCCGGATTT**G**C

3.4 Discussion of Results

Dickeya solani has only recently been described as a novel species although it is believed to account for the increase in blackleg and soft rot disease on potato since its emergence in 2005 (van der Wolf *et al.*, 2014). Knowledge regarding the ecological and genomic traits which facilitate the success of *D. solani* as a plant pathogen has been limited therefore research has begun to focus on understanding the genetic characteristics of *D. solani* which may account for its pathogenesis. Molecular techniques have included the MLSA and pyrosequencing methods used in this study, in addition to variable number tandem repeat (VNTR) work carried out at Fera (Parkinson *et al.*, 2015). In this research, the genetic relationships between *D. solani* strains were studied using two sequencing techniques. Both pyrosequencing and MLSA defined the relationships between *D. solani* species but at varying levels of resolution.

Identification of *Dickeya solani* has been based on sequencing of either the *recA* or the *dnaX* gene (Parkinson *et al.*, 2009; Sławiak *et al*, 2009). Using a single gene has its limitations as not all strains are identical with all members of the species. In addition, gene duplication or loss, horizontal gene transfer, and recombination can all interfere with the accurate construction of the evolutionary history of the organism so phylogenetic analysis is not recommended based on a single gene (Feil *et al*., 2001; Ochman *et al.*, 2000; Parkinson *et al.*, 2009). The use of multiple genes is necessary for accurate identification of strains, making MLSA ideal for strain identification which incorporates the analysis of several genes and gives a clearer picture of relationships and relatedness between strains (Brady *et al.*, 2008; Menna *et al.*, 2009; Young *et al*., 2008).

The MLSA research in this study was based on previous work at SASA focussing on characterising *D. dianthicola* and also utilised the seven housekeeping genes from the *D. dianthicola* study (*recA, dnaN, fusA, gapA, purA, rplB, rpoS* and *gyrA*), in addition to two more genes*: dnaX* and *dnaJ*. Both *dnaX* and *dnaJ* were identified through computational analysis by Zielger (2003) to be useful predictors of genome relatedness and valuable for the discrimination of bacterial species. *dnaX* has been previously used in phylogenetic studies of *Dickeya* species in addition to *recA* and *dnaJ* has been useful in studies of other members of the *Enterobacteriaceae* (Hong *et al*, 2007; Dauga, 2002; Sławiak *et al.*, 2009). Based on *dnaX* and *dnaJ* phylogenetic analysis, *D. solani* is separated into a distinct clade from other *Dickeya* species; however, MK10 and MK15 fell slightly out with this clade. MK10 continues to vary from other strains when

pyrosequencing of SNPs was carried out but both MK10 and MK15 fall within the homogenous clade when all sequences are concatenated which supports their identification (which had been carried out using *recA* sequencing in a previous study) as *D. solani*.

The concatenated sequences readily distinguish *D. solani* from other members of the *Dickeya* genus and recover the *D. solani* isolates in a homogenous clade. This clade was found to be more closely related to *D. dadantii* subsp. *dadantii* and *D. dadantii* subsp. *dieffenbachiae* with *D. dianthicola* and *D. paradisiaca* being most distant from the *D. solani* clade. Individual phylogenetic studies of each gene also show similar clustering *of D. solani*. The clustering of *D. solani* into a single clade, based on the concatenated sequences suggested that there was little difference between the *Dickeya solani* strains and that it is essentially a clonal pathogen.

The use of the data procured through the MLSA study has facilitated the publishing of an online database which can be accessed and used by other researchers to characterise new *Dickeya* strains. The development of the online database is important for knowledge transfer and allows for distinguishing between pathogens that cause similar disease symptoms (Almeida *et al.*, 2009). Like the real-time assay, described in Chapter 2, this database allows for a more time sensitive and streamlined method for the identification of *D. solani*. Rather than requiring additional strains and needing to assemble phylogenetic trees, gene sequences from a single isolate can be compared within the database and the *Dickeya* species identified based on the sequence. Additional sequences can be submitted to the database which can aid with mapping and monitoring the spread of the pathogen as, in addition to the sequence information, information regarding the host and geographical isolation is recorded.

The data from the MLSA analysis was used to classify *D. solani* as a novel species within the *Dickeya* genus, specifically the sequence analyses based on the genes *dnaN, fusA, gapA, purA,* and *rplB* (Van der Wolf *et al.*, 2014). Van der Wolf *et al.* (2014) also carried out analysis of *D. solani* isolates using Pulsed-Field Gel Electrophoresis (PFGE) after restriction digestion by *Xba*I or I-*Ceu*I and found that all strains of *D. solani* clustered in a tight, distinct group that was well separated from the other taxa. The clustering of *D. solani* as seen in the MLSA and PFGE analysis suggest that the isolates can be considered a single clone (van der Wolf *et al.*, 2014). It is not uncommon for highly specialised pathogens, existing in close association with the host, to show a high

degree of clonality and the close association of *D. solani* can be accounted for by its recent emergence or it is possible that there has yet to be an opportunity for genetic exchange (Tibayrenc & Ayala, 2012; Spratt, 2004).

Based on the concatenated sequences assembled in the MLSA study, there is no variation between the *Dickeya solani* strains regardless of their geographical location and host; however, MK10 and MK15 show slight variation in their *dnaJ* and *dnaX* assemblies. This indicates the potential for Single Nucleotide Polymorphisms (SNPs) which will also permit higher resolution characterisation of *Dickeya solani*. Using SNP analysis as a form of fingerprinting has potential to assist outbreak studies and tracking. Unlike Sanger sequencing in which the targeted sequence to be read is roughly 20-30 bases from the sequencing primer, pyrosequencing can generate sequencing signals immediately downstream from the primer and sequencing can begin at the base next to the annealed primer which makes primer design much easier and more flexible (Gharizadeh *et al.*, 2007). The process of pyrosequencing single-stranded DNA takes only 15 minutes compared to Sanger sequencing which can take up to four hours (Gharizadeh *et al.*, 2007).

In this study eight SNP markers were used to characterize 14 strains of *D. solani.* Based on SNPs, *D. solani* was separated into two main groups with four strains that did not fall into either group. MK10, isolated from a rotten tuber in Israel, did not fall into either group and also did not fall into the *D. solani* clade in the MLSA using *dnaJ* and *dnaX*. The significance of this difference is unclear and further genomic analysis would be required; however, MK10 has been completely sequenced and its sequence used in this study to design the pyrosequencing primers. These SNP markers can be used in the future to carry out further SNP analysis through pyrosequencing and facilitate the monitoring of the spread and evolution of *D. solani.*

Pyrosequencing has the potential to be an informative complement for screening for *D. solani* outbreaks and benefits from a short time to obtain results, an ease of use, flexibility of the analysis and the detection sensitivity. This is supported by subsequent research by Khayi *et al.* (2015) which separates *D. solani* into a distinct clade based on MLSA of 11 housekeeping genes (*rpoD, gyrB, recA, rpoS, dnaX, dnaA, gapA, fusA, rplB, purA,* and *gyrA*). In addition, their research also used SNP analysis with Illumina technology and separated *D. solani* into three groups based on SNP variations. The research carried out at Fera based on variable number tandem repeat (VNTR) showed

that little variation is seen between *D. solani*, with only three profiles identified and only one profile showed any variation (Parkinson *et al.*, 2015).

Genomic analysis of *Dickeya solani* has been carried out to a higher resolution through the use of MLSA and the identification of SNPs; although *D. solani* variability remains limited. Further analysis, particularly using SNPs, presents the potential to monitor the genetic evolution of *D. solani* and track any potential genetic variations that may emerge. This research suggests that the success of *D. solani* is due to its spread and the international trade in potato as there is little evidence of genetic variation between isolates with disease being caused by a single strain.

Chapter 4. Transmission of *Dickeya solani* from Infected Seed Tubers under Scottish Conditions

4.1 Introduction

4.1.1 *Importance of the Global Potato Industry and the Influence of Potato Pathogens*

Potato, the world's fourth-largest food crop, has become a fundamental part of the global food supply. International potato trade has doubled in volume and increased almost four-fold in value since the 1980s even though only 6% of the global potato crop is traded internationally (FAO, 2008). A major limiting factor on potato production is its vulnerability to disease: potato diseases have a significant impact on trade and challenges come from the diseases being dynamic and new diseases constantly emerging.

Significant losses due to potato diseases can occur at all stages of production: late blight, caused by *Phytophthora infestans*, represents the biggest single production constraint and affects yield and can destroy entire crops in as little as 10 days. Late blight was the cause of the Irish Potato Famine in the 1840s and remains a problem today, demonstrating the significance of understanding and implementing effective controls of potato disease (Dehnen-Schmutz *et al.*, 2010). Black scurf, caused by *Rhizoctonia solani*, disfigures the tubers thereby reducing their market value and infection by *Dickeya* sp. and *Pectobacterium* sp. can affect the potato at every stage, from field, storage and transit (Tsror *et al.*, 1999). Losses due to *Dickeya* and *Pectobacterium* spp. are due to the ability of the pathogens to infect potato tubers, break down and utilise the pectin in the tubers for energy, causing soft rot, or through infection of the potato plant stems leading to blackleg which is seen during the growing season (Pérombelon and van der Wolf, 2002). In many cases, the only way to control disease is to prevent its introduction; typically achieved by using disease-free seed potatoes and ensuring the growing environment is disease-free. Potatoes are vegetatively propagated, that is, they are asexually reproduced from one plant and the offspring are genetically identical to the mother plant, which can facilitate the introduction of pathogens to offspring, can spread the disease from generation to generation. In an attempt to control this, the number of generations for seed production is restricted as successive cycles of vegetative propagation can increase the severity and incidence of pathogens in planting material (Pérombelon *et al.*, 1980; Tsror *et al.*, 1999; Thomas-Sharma *et al*., 2016).

4.1.2 *Dickeya spp. and Pectobacterium spp.: Their Significance to Potato Production*

Blackleg and soft rot are the major causes of potato seed downgrading and rejections in Northern Europe and are likely to become more significant in the coming decades, especially disease caused by *Dickeya* spp. and *Pectobacterium* spp. due to the complexity of trade and climate change (Degefu *et al*., 2013). *Pectobacterium* spp. and *Dickeya* spp. are the major causative agents of blackleg and tuber soft rot of potato and, alongside bacterial wilt caused by *Ralstonia solanacearum* and ring rot and common scab caused by *Clavibacter michiganensis* subsp. *sepedonicus* and *Streptomyces scabiei*, respectively, are the most economically important bacterial diseases in seed potato production (Pérombelon & Kelman, 1980; Pérombelon, 2002; van der Wolf & De Boer, 2007; Czajkowski *et al*., 2011). *Pectobacterium* spp. and *Dickeya* spp. are very difficult to control once they have entered the crop. Some *Pectobacterium* spp. and *Dickeya* spp. have a wide host range making dissemination easier, and certainly the emergence of *D. solani* as a potato pathogen is believed to have originated in a field of ornamental bulbs and then spread into potato and from where it was able to spread internationally (Sławiak *et al.*, 2009; Parkinson *et al.*, 2009). *Dickeya solani*, which emerged in continental Europe in 2005/2006, is highly aggressive and in less than a decade has become the predominant cause of blackleg in many European countries. It was detected in England in 2007 and in Scotland in 2009. The infection in Scotland only ever found on potatoes grown from European-sourced seed (Toth *et al.*, 2011). It has generally been accepted that *Dickeya* and *Pectobacterium* infection is spread through latently infected seed tubers, and this is the main infection route, facilitating the spread of the pathogens over long distances, and is of particular significance because of the global trade in seed potatoes (Pérombelon, 1974; Czajkowski *et al*., 2009). It is possible for the pathogens to then spread through the vascular tissue of the plant through the roots and once in the stem can either cause blackleg or remain in a latent form (Czajkowski *et al*., 2011). Bacteria can be transmitted directly from the mother tuber via the plant vascular tissue into the stems, stolons and progeny tubers (Hélias *et al.*, 2000; Czajkowski *et al.*, 2010). Factors that promote the spread of *Pectobacterium* and *Dickeya* spp., in addition to increased presence of the pathogen on seed, are higher moisture levels, resulting from poor soil drainage, over-irrigation and wet spring weather. Temperature can also play a role with certain species of *Dickeya* and *Pectobacterium* favouring either warmer or cooler conditions (Tsror *et al.*, 2006; Toth

et al., 2011). The increase in virulence which is seen with increased moisture levels is considered to be due to anaerobic conditions induced by water coverage of the tubers which restricts the oxygen-dependant resistance factors (Pérombelon *et al.*, 1989). Irrigation and rainfall have a direct impact on the moisture of the soil and foliage and have been proven to increase the development of soft rot symptoms and can assist with the movement of bacteria through the plant's vascular tissue due to the flow of water evapotranspiration which is also influenced by temperature (Pérombelon, 2002; Tandogdu and Camgoz, 1999; Gill *et al.*, 2014).

Soil-borne *Dickeya* spp. infect potato roots from where the bacteria may further colonise the plant including the progeny tubers. Bacteria enter the roots of the infected plants via wounds caused by other soil-borne pathogens and pest organisms or natural openings which arise during lateral root formation (Czajkowski *et al.*, 2010). Studies have shown that pathogen-free seed lots can become infected with blackleg and stem rot-causing bacteria within a few generations in the field. In the Netherlands, minitubers became infected within two field generations and 17 out of 50 seed lots were found to be contaminated with *Dickeya* sp. when tested using enrichment PCR (van der Wolf *et al.*, 2009).

4.1.3 *Temperature and the Spread of* Dickeya *spp. and* Pectobacterium *spp.*

Both *Dickeya* and *Pectobacterium* spp. cause blackleg and tuber soft rot and the most important factor influencing which pathogen dominates is temperature (Pérombelon & Hyman, 1986; Toth *et al.*, 2011). Depending on the bacteria spp., growth can be seen at temperatures ranging from 21^{o}C to 37^{o}C, and it is generally accepted that disease at temperatures above 25^{o}C is largely caused by *Dickeya* spp. and below 25^{o}C by *Pectobacterium* spp. (Lumb *et al.*, 1986; Toth *et al*., 2011). Variability of growth has been shown within the species themselves, with certain *D. dianthicola* stains showing optimal growth temperatures of between 21^{o}C and 25^{o}C (Toth *et al.*, 2011). It is hypothesised, and generally accepted, that the aggressiveness of *D. solani* correlates with temperature; accordingly, as the temperature increases, so does aggressiveness (Laurila *et al.*, 2008; Lojkowska *et al.*, 2010; Czajkowski *et al*., 2012). Unfortunately, comparison studies are limited, especially as studies have used a variety of temperatures and conditions; for example, field studies in Israel have used tubers and day temperatures of 28-30^{o}C and overnight temperatures of 22-24^{o}C, in Spain, tubers experienced daytime temperatures of 28^{o}C and overnight temperatures of 18^{o}C, and in

Finland temperatures were between 21-23^{o}C (Tsror *et al.*, 2009; Palacio-Bielsa *et al.*, 2006; Laurila *et al*., 2008). Growth of *D. solani* has been seen at temperatures up to 39^{o}C in both glasshouse studies, where it has been determined to be more aggressive than *D. dianthicola*, and in the field conditions of Israel where temperatures were high (Laurila *et al*., 2008; Sławiak *et al*., 2009; Tsror *et al*., 2009). Conversely, at lower temperatures, such as those experienced during field studies in Finland, there were greater incidences of disease caused by *D. dianthicola* (Laurila *et al.*, 2008).

4.1.4 *Spread of* Dickeya *spp. from Planted Seed Tubers*

Previous field studies using vacuum infiltration to inoculate seed tubers resulted in the progeny tubers being infected with *Dickeya solani* at their formation; which suggest that *Dickeya* sp. move readily through vascular tissue in the growing plant resulting in infection of the progeny tubers (Czajkowski *et al.*, 2010). Other studies have shown that *D. solani* is capable of colonising the roots of potato plants from inoculated soil within one day and after 15 days, *D. solani* was found in the stolon and stems of potato (Czajkowski *et al.*, 2010). A study carried out by Czajkowski *et al*.(2010), which inoculated soil with *D. solani* three weeks after the planting of tubers by immersion in a suspension of 10^{8} CFU.ml^{-1} in water, detected *Dickeya* spp. inside 42% of roots, 13% of stems and 12% of stolons with intact roots two weeks after soil inoculation and in plants with intact roots, *Dickeya* sp. was detected inside 50% of roots, 25% of stems and 25% of stolons. Symptoms of blackleg were expressed 30 days after soil inoculation. Czajkowski *et al*. (2010) were able to visualise the spread of *Dickeya* spp. from root inoculation to systemic colonisation of the potato plant using a *D. solani* tagged with green fluorescent protein (GFP). The study showed that infection happened rapidly with internal colonisation beginning as early as one day after soil inoculation. Within one month, *D. solani* could be visualised within the stolons and progeny tubers through dilution plating, epifluorescence stereo microscopy (ESM) and confocal laser scanning microscopy (CLSM). The study suggested that *D. solani* shared the infection mechanism of other root-invading pathogens and that colonisation occurs over three stages: *D. solani* colonise the surface of roots before penetrating the roots and establishing infection within the cortex before moving into the parenchyma cells of the pith and xylem vessels of the stems. From this location it was judge to be relatively easy for them to spread to other locations within the plant (Czajkowski *et al.*, 2010).

4.1.5 *Specific Aims for Understanding the Transmission of* Dickeya solani.

With emerging diseases, avoidance is an important method of control; therefore, preventing introduction is key especially as *Dickeya* and *Pectobacterium* spp. are difficult to control once in a potato crop. In potatoes, infection into new environments is generally through the movement of asymptomatically infected seed (Pérombelon, 1974; Pérombelon and Kelman, 1980). *Dickeya solani* has spread rapidly across Europe since its emergence in 2005; therefore it is vital that trade does not allow it to spread to other potato growing regions of the World (Laurila *et al.,* 2008; Lojkowska *et al.,* 2010). *Dickeya solani* has only ever been found in a small number of ware crops grown in Scotland in 2009 and 2010 and in all cases the infection could be traced back to infected, imported seed. Over the past decade, trade in certified British seed potatoes has increased by 30%; with over 103,000 tonnes exported in the 2013/2014 season, of this, Scottish exports made up the majority; almost 80,000 tonnes (ADHB, 2015; SASA, 2015). In order for Scotland to maintain its reputation for producing consistently high health and high quality seed potatoes understanding whether *D. solani* can spread under Scottish environmental conditions is vital; consequently, one focus of this study was to determine whether transmission from mother to daughter tubers and transmission between infected and healthy plants under field conditions was possible under prevailing conditions.

4.2 Materials and Methods

4.2.1 *Infection and Planting of Tubers*

The experiment was carried out over three growing seasons in 2010, 2011 and 2012, using the *Dickeya solani* isolate MK13 throughout. MK13 was originally isolated from a ware import into a Scottish packing plant from Israel. The isolate was maintained at -80ºC on cryovial beads and revived on CVPM at 36ºC for 48 hours. Details of all reagents and media used can be found in Appendix 2. Cultures were re-isolated onto Nutrient Agar for 24 hours at 36ºC. Colonies from the Nutrient Agar plates were added to sterile distilled water to an optical density of 0.2 at 600 nm (OD_{600}) to give an approximate concentration of 10^8 colony forming units (CFU) per ml. Dilutions were made using autoclaved water which had been purified using reverse osmosis (Thermo Scientific) to give working concentrations of 10^5 $CFU.ml^{-1}$. The potato cultivar Nicola was used throughout this study as previous studies in Israel had shown it to be susceptible to *D. solani* infection (Tsror *et al*., 2009). In 2012 the cultivar Hermes was also included alongside Nicola with 90 cv. Hermes and 180 cv. Nicola seed tubers planted.

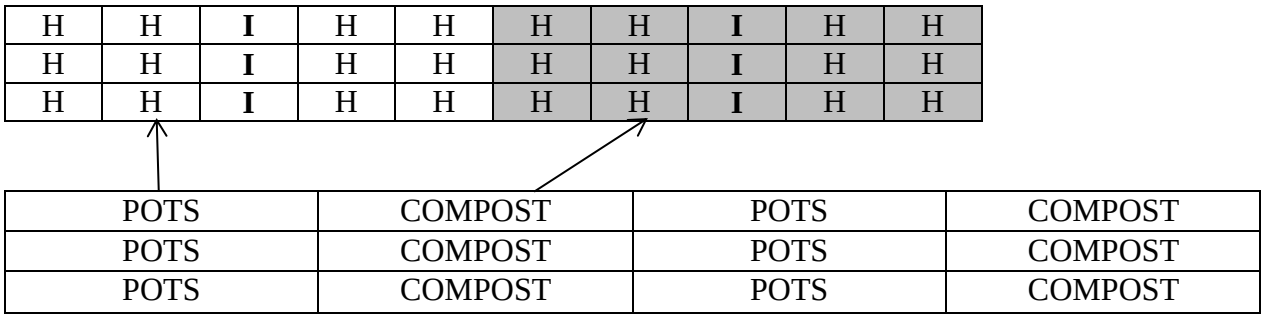
Tubers were initially washed in tap water to remove excess soil and debris, allowing for access to the skin surface. Washed tubers were stored overnight in autoclave bags at a warm temperature (approximately 25ºC) to facilitate opening of the lenticels. The following day, tubers were grouped in net bags for vacuum infiltration. Tubers were exposed to either a suspension of 10^5 $CFU.ml^{-1}$ *Dickeya solani* or a sterile water control and held under vacuum at -80kPa for 15 minutes at room temperature before being removed. The total amount of suspension was 10L. The suspensions were prepared from overnight cultures into water which had been sterilised under UV light and diluted freshly prior to incubation. Once infiltrated, the tubers were left in the net bags overnight to dry. Control (water) tubers (i.e., healthy tubers) were infiltrated first to avoid contamination and were kept separate from tubers infiltrated with the pathogen to avoid cross-contamination. Due to Scottish legislation tubers inoculated with *D. solani* were planted within three rows of raised beds in the quarantine area of SASA as shown in Figure 4.1**.** Tubers were planted in either pots with holes in the base, which were then embedded in peat, or directly in compost in the raised beds, the layout of which is indicated in Figure 4.2. Healthy tubers were planted first, in the last week of April, followed by the inoculated tubers. All plants were watered heavily to ensure plenty of

moisture was provided throughout the growing season. Plants were inspected weekly after emergence for blackleg and wilting symptoms.

Figure 4.1 Layout of Raised Bed Experiments for Studying Transmission of *Dickeya solani* under Environmental Conditions using cv. Nicola

Figure 4.2 Layout of Seed Tubers Planted in the Raised Beds at SASA.

Each raised bed contained 60 plants. Thirty were planted in pots and 30 were in compost. In total there were 180 seed tubers planted.



Healthy seed tubers are denoted with H

Infected tubers are denoted as I.

4.2.2. *Harvesting and Testing of Tubers*

This experiment was designed to determine the efficiency of *Dickeya solani* as a pathogen by monitoring spread from infected mother tubers to daughter tubers and also monitoring the spread from inoculated to uninoculated plants grown in a continuous bed or contained within pots sunk into a peat bed.

Potato progeny tubers were harvested in the first week of October of the growing season, by hand. The week prior to harvesting, the plants were treated to senesce the haulm. Seed from healthy plants had their progeny harvested first to avoid potential contamination from the infected tubers. Tubers from each plant were collected into brown paper bags and grouped according to the number of the mother tuber and stored at 4^{o}C prior to testing for *Dickeya solani*.

Individual tubers from inoculated seed were counted and processed individually. Tubers from healthy seed were cored at the stolon end, aggregated and processed collectively for each plant. Tubers were processed as harvested, including any soil/dirt that remained on the surface.

Infected tubers were sampled by taking one core from each individual tuber at the stolon end and placing in Falcon tubes containing 20ml of extraction buffer then incubating with shaking at 36^{o}C overnight. The overnight suspension was vortexed and 1ml removed and diluted to 10^{-6} using Ringer's solution. One hundred microliters of each dilution was then spread onto CVPM and incubated at 36^{o}C for 48 hours. Pit-forming colonies were re-isolated onto Nutrient Agar and incubated at 36^{o}C for 24 hours. Colonies grown on Nutrient Agar were removed using a sterile loop and added to 1ml of sterile distilled water contained within a 1.5ml Eppendorf tube and boiled at 100^{o}C for 5 minutes. One microlitre of boiled cells was used in the 'Nassar Assay' as described in Appendix 2 (Nassar *et al.*, 1996). Samples found to be positive after the 'Nassar Assay' were sequenced based on the *recA* gene according to Chapter 3. Pit-forming samples were also used to test the SOL-C and *fusA* assays (Appendix 2).

4.3 Results from Transmission Experiments of *Dickeya solani* from Mother to Daughter Tubers and from Infected to Healthy plants

Testing of inoculated cv. Nicola seed tubers in the growing seasons of 2010, 2011 and 2012 showed that not all inoculated tubers produced infected progeny tubers or symptomatic plants and over the three years, the proportion showing infected progeny tubers varied. In 2010 11 out of 36 inoculated tubers (30.5%) produced infected progeny, in 2011, 17 of the 36 inoculated tubers produced infected progeny (47.2%) and in 2012 the number was 2 out of the 54 (5.6%). As only *D. solani* had been used for the inoculation, it was the only pathogen tested for within the samples.

Symptom expression that could be attributed to blackleg disease such as wilting, stem rot and chlorosis of leaves was variable over the three year period. Results in Table 4.1 show that in 2010, four of the 11 plants with infected progeny (36.4%) showed symptoms, in 2011 eight out of 17 plants with infected progeny (47.1%) expressed symptoms and in 2012, both (100%) plants with infected progeny showed symptoms. In all cases, symptoms could be attributed to the presence of *Dickeya solani*.

Results from post-harvest testing of plants grown from uninoculated tubers, shown in Table 4.2, demonstrated transmission to only one plant out of 144 (0.7%) in 2010; this plant expressed symptoms of infection and was grown directly adjacent to a plant grown from an infected mother tuber and grown in compost. In 2011, four out of the 144 un-inoculated plants (2.8%) were positive for *Dickeya solani*. None of the plants expressed symptoms and all four plants were grown directly adjacent to plants grown from inoculated plants with two grown in compost beds and two grown in pots which were embedded in peat. No infection was found in the uninoculated plants in 2012.

In contrast, the results from the small study in 2012 using cv. Hermes as indicated in Table 4.3 showed that out of the 18 inoculated mother tubers, ten produced plants with progeny tubers that tested positive for *Dickeya solani* (55.6%); although only four of the ten *Dickeya solani*-positive plants from inoculated tubers expressed symptoms of disease (40%). None of the uninoculated had progeny tuber infected with *D. solani*.

Combining the results over the three year period, *Dickeya solani* infection was observed in the minority of plants and from the plants positive for infection, few presented

symptoms. Of the total 540 cv. Nicola seed tubers planted, 35 plants were identified as producing progeny tubers identified as positive for *D. solani*: 30 were grown from inoculated seed tubers and five from uninoculated seed tubers. The study involving cv. Hermes produced a higher number of offspring infected with *D. solani* with ten of the plants grown from 18 infected seed tubers testing positive for *D. solani*. Similar to the results seen with cv. Nicola, not all of the infected plants were symptomatic.

Weather data was gathered the Met Office Weather Station at Gogarbank, Edinburgh (Met Office station 03166). Summaries of daily maximum and minimum temperatures are shown in Table 4 and comparison of the maximum temperatures shown in Figure 4.3. The highest maximum daily temperature, 25.7^{o}C, was in 2011. Interestingly, when the temperature was higher, as in 2011, more *D. solani* was found in progeny tubers and the wetter the season, the more symptoms were expressed by the progeny tubers that were infected with *D. solani*, as seen in 2012.

Table 4.1 Results from the Raised Bed Experiments Carried out at SASA from 2010-2012 Using cv. Nicola tubers Inoculated with *Dickeya solani.*

Growing season	2010	2011	2012
Number of tubers inoculated	36	36	36
Number of plants from inoculated seed tubers with infected progeny tubers	11	17	2
Number of plants from inoculated seed tubers expressing symptoms	4	8	2

Table 4.2 Results from the Raised Bed Experiments Carried out at SASA from 2010-2012 Using Healthy cv. Nicola Tubers

Growing season	2010	2011	2012
Number of uninoculated tubers planted	144	144	144
Number of plants from uninoculated seed with infected progeny tubers	1	4	0
Number of plants from uninoculated seed with infected progeny tubers expressing symptoms	1	0	0

Table 4.3 Results from the Raised Bed Experiments Carried out at SASA in 2012 using cv. Hermes Tubers.

	Inoculated seed tubers	Uninoculated seed tubers
Number of tubers planted	18	72
Number of plants with infected progeny tubers	10	0
Number of plants with infected progeny tubers expressing symptoms	4	0

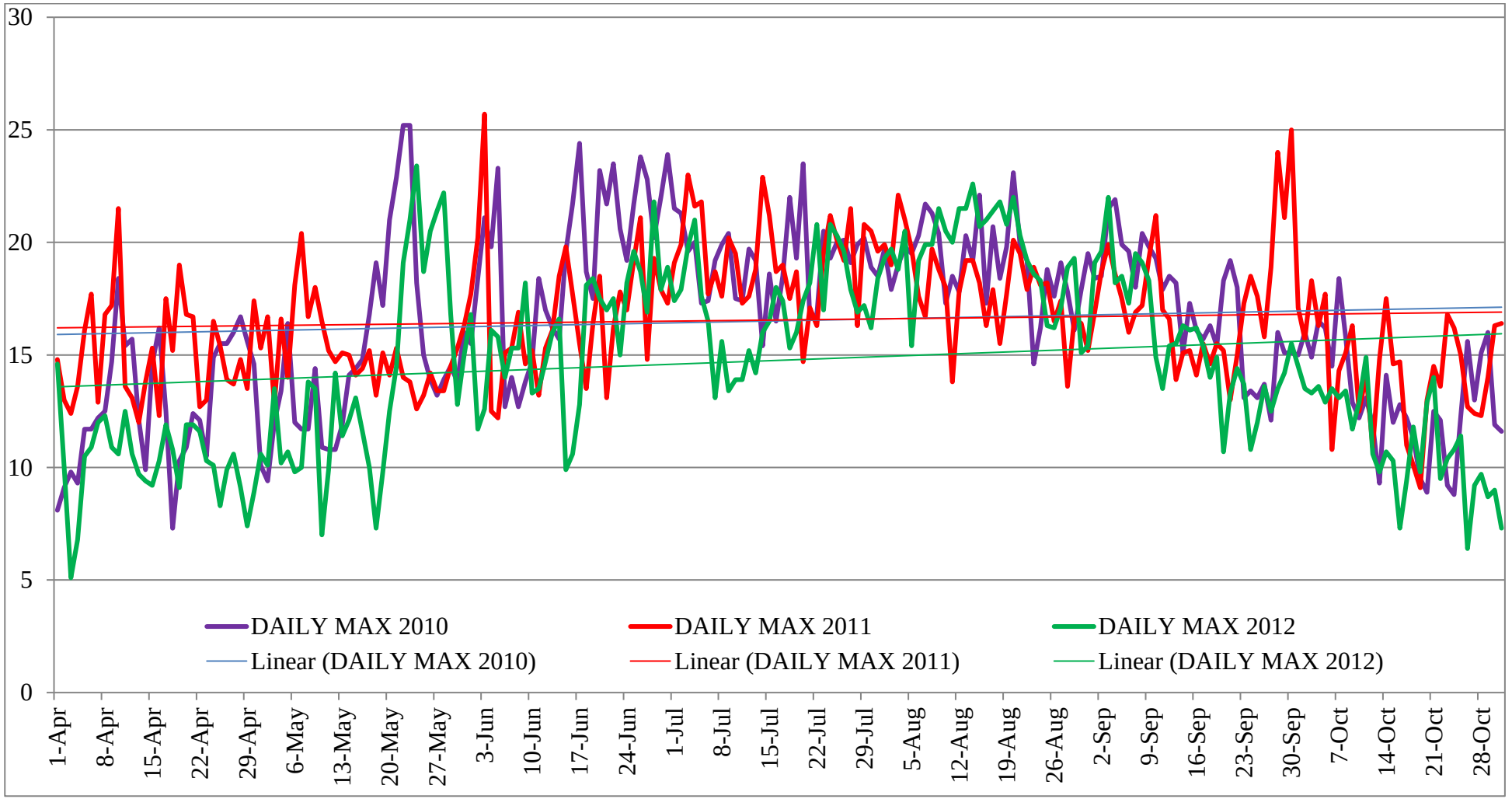


Figure 4.3 Graph Showing the Daily Maximum Temperatures from April to October Over the Three Growing Seasons: 2010-2012.

4.4 Discussion

4.4.1 *Understanding the Transmission of* Dickeya solani *in Seed Potatoes*

The emergence of *Dickeya solani* in the past decade, its rapid spread and dominance as a blackleg-causing pathogen in Europe and Israel has raised concern and is now considered an important pathogen in seed potato production worldwide (Pérombelon & Kelman, 1980; Czakjowski *et al.*, 2011). Assumptions of the transmission of *D. solani* are based on the mechanisms of other blackleg and soft-rot causing pathogens such as *Pectobacterium atrosepticum*; consequently, the spread of *D. solani* is believed to be facilitated by the international trade in seed potatoes; therefore, control measures require a holistic approach, beginning with the use of pathogen-free seed tubers (Lumb *et al*., 1986; Sławiak *et al*., 2009a; Tsror *et al*., 2009; Czajkowski *et al.*, 2011).Studies have shown that even when using pathogen-free propagation material initially, within two field generations *D. solani* was present in approximately 30% of harvested tubers; however the mechanism for the introduction of *D. solani* is unknown and the spread from infected tubers is poorly understood (Toth *et al.*, 2011). The aim of this study was to better understand the spread of *D. solani* by studying its dissemination from infected seed tubers to progeny tubers, combined with assessing the potential for spread from infected tubers to neighbouring progeny tubers grown from healthy seed.

The ability of *Dickeya* spp. to spread and cause disease depends on a variety of factors including: the amount of inoculum present, the potato cultivar, temperature and humidity/moisture levels (Toth *et al.*, 2011). Temperature in particular is believed to play an important role, especially in the virulence of the pathogen (Gill *et al.*, 2014). In *Pectobacterium atrosepticum*, the concentration levels of bacteria in the mother tuber influences the speed of mother tuber rotting initiation and with *Dickeya* spp. being more aggressive, it is possible that population density plays a smaller role (Bain *et al.*, 1990; Czajkowski *et al*., 2011).

As part of project R437 funded by the Potato Council, a study was carried out by Fera, England, simultaneously to the study in Scotland, with the same objective of assessing the spread of *Dickeya solani* from inoculated mother tubers to daughter tubers and between neighbouring plants. SASA provided Fera with vacuum-infiltrated tubers at three inoculum levels: high (10^7 CFU.ml^{-1}), medium (10^5 CFU.ml^{-1}) and low (10^3 CFU.ml^{-1}). Seed was planted in May and harvested in September. In 2010 and 2011 no

progeny that originated from uninoculated seed showed symptoms of blackleg. In 2012, blackleg was observed on tubers from uninoculated seed and was determined to be caused by background *Pectobacterium atrosepticum* which was isolated from the symptomatic stems rather than the tubers. Aerial blackleg was also present in the growing season of 2012 in the Fera field studies and attributed to the presence of wind damaged stems. Due to the unusually wet conditions throughout the growing season of 2012, the study at Fera was cut short. As at SASA, Fera utilised the real-time assays described in Chapter 2 to identify the causative pathogen of blackleg symptoms and *D. solani* was identified in progeny tubers at all three levels of original inoculum.

4.4.2 *Pathogen and Inoculum Levels*

This study utilised the *Dickeya solani* isolate, MK13, isolated in Scotland from a ware import from Israel which had been used previously in transmission studies carried out at SASA focussing on *D. dianthicola* transmission in comparison to that of *D. solani* and *Pectobacterium atrosepticum* which were carried out in 2010 (G. Cahill, SASA, unpublished data).

The inoculum level on seed tubers are considered to have a role in symptom development and the inoculum levels of 10^5 CFU.ml^{-1} was chosen as previous studies on the symptom development of *Pectobacterium atrosepticum* disease found that vacuum infiltration of bacterial suspensions estimated to be between 4.5 x 10^8 CFU.ml^{-1} and 4.5 x 10^6 CFU.ml^{-1} were found to cause earlier and more severe development of symptoms and the inoculum concentration was found to affect the proportion of plants in all classes of disease symptoms and the highest inoculum concentration resulted in the highest rate of non-emergence (Hélias *et al.*, 2000). Earlier studies by Lapwood and Read (1985) also used inoculations of *P. atrosepticum* of 10^6, 10^7 10^8 cells.ml^{-1} to assess the susceptibility of potato cultivars to the pathogen. In order to achieve an adequate volume of bacterial suspension, a concentration of 10^5 CFU.ml^{-1} was chosen for our experiments, in addition, to assuming that at such a concentration, tubers would not completely rot in the field and would allow for sampling of potato matter to isolate *D. solani*. The study carried out at Fera used tubers inoculated with three different bacterial loads and over the three growing seasons it was observed that the incidence of blackleg directly correlated with the original bacterial load and blackleg symptoms were observed at all three original inoculum levels, including the 10^5 CFU.ml^{-1} as used in the SASA study.

With vacuum infiltration, the seed tuber may rot at any time over the growing season; in Scotland this often occurs during July (Pérombelon, 1976; Lapwood & Read, 1985). In addition, unpublished in vitro studies found this pathogen to cause disease at the levels used in this study in potato slice assays at 36ºC (Toth, unpublished data). Fera carried out a study as part of the larger Potato Council Project R437 assessing the expression of blackleg symptoms from varying inoculum levels of *Dickeya solani* at either 21ºC or 27ºC, using cv. Maris Piper tubers provided by SASA. The seed tubers were inoculated from a liquid suspension of either: 10^1, 10^2, 10^3 or 10^4 CFU.ml^{-1} and incubated at 21ºC or 27ºC for six days. *Dickeya solani* was shown to produce a greater amount of rot than *Pectobacterium atrosepticum* and the levels of rot observed were found to be lower with original concentrations of 10^1 and 10^2 CFU.ml^{-1} compared with 10^3 and 10^4 CFU.ml^{-1}; although the levels of rot present at 21ºC was not considered to be significant. The amount of rot produced when tubers were originally inoculated with levels of 10^3 and 10^4 CFU.ml^{-1} was higher by both pathogens when incubated at 27ºC. Although the cultivar of seed tuber used was different to that used in the field studies, the Fera study supports the use of initial inoculum levels of 10^5 CFU.ml^{-1} to be sufficient to cause disease.

4.4.3 *Potato Cultivar*

Potato cultivar is another factor in disease development, although previous work on developing potato cultivars that are resistant to blackleg has had limited success and cultivars that are completely resistant to *Dickeya* and *Pectobacterium* spp. have not been developed (Lapwood *et al*., 1984; Lapwood & Harris, 1982; Czajkowski *et al*., 2009). Data on the susceptibility of certain potato cultivars to *Dickeya* spp. is limited and is based on studies in Israel (Tsror *et al*., 2009; Toth *et al*., 2011). The cultivar Nicola was chosen as it had shown previous susceptibility to *D. solani* infection in experiments carried out in Israel (Tsror *et al*., 2009); however, it should be recognised that environment may also play a part in disease severity. Based on personal communication (Tsror; unpublished data), the cultivar Hermes was subsequently included in 2012 and 10 out of 18 (55.6%) infected mother tubers produced infected daughter tubers which was higher than observed in all the studies with cv. Nicola. As with cv. Nicola transmission is inefficient and no transmission was observed to adjoining inoculated plants. In the Netherlands, studies on the distribution and population of blackleg used naturally infected potatoes of the cultivars Arcade and Konsul from the field which were infected with both *P. carotovorum* subsp.

carotovorum and *Dickeya* sp. (Czajkowski *et al.*, 2009). Researchers found that high concentrations of *Dickeya* spp. were found at the stolon ends of the tubers, with lower densities found in the peel and deeper located potato tissue. The two naturally infected seed lots were not found to be infected with *Pectobacterium* spp. In 2010 and 2011 Fera studied the incidence of blackleg between different varieties of the ten most commonly grown potato cultivars and determined that host genotype was unlikely to play a role in influencing blackleg development due to the changing susceptibility depending on the growing conditions.

4.4.4 *Temperature and Weather*

Temperature is considered to be the most important factor in determining which pathogen will dominate and cause disease in potato plants and the relative aggressiveness of *Dickeya* spp. is linked to temperature (Toth *et al*., 2011). Studies have shown *D. solani* to be capable of causing disease at temperatures ranging from 21-39ºC but when temperatures exceed 25ºC it is more likely for disease caused by *Dickeya* sp. to develop with *Pectobacterium atrosepticum* more dominant at temperatures below 25ºC (Lumb *et al.*, 1986; Palacios-Bielsa *et al.*, 2006; Laurila *et al.*, 2008; Sławiak *et al.*, 2009b; Toth *et al.*, 2011; Tsror *et al.*, 2009). The temperatures in this study ranged from -3.8ºC to 25.7ºC. The maximum temperatures recorded in over the three years were 25.2ºC, 25.7ºC and 23.4ºC respectively which are below the optimal temperatures used in previous studies, and below the 27ºC that was determined in the study carried out at Fera to result in significant rot. The growing season of 2011 produced the largest proportion of infected tubers (17 plants grown from infected seed and four plants grown from healthy seed); and had the highest recorded temperature (25.7ºC). Over the three years the overall temperatures were sub-optimal when compared to conditions frequently observed on continental Europe and may also explain why transmission was not always observed. The 2012 growing season, which had the least number of plants with infected progeny tubers (two in total), only reached a maximum temperature of 23.4ºC. Overall, the average temperatures during the growing season in Scotland between 2010 and 2012 did not appear to reach temperatures that are seen in other countries with a larger problem with *D. solani.* In particular, temperatures in the Netherlands for the growing season (April – October) of 2010 ranged from a minimum of 1ºC to a maximum of 33ºC, with an average of 14ºC. For the growing season of 2011 temperatures ranged from 2ºC to 32ºC, with an average temperature of 15ºC and for 2012, the temperature ranged from -1ºC to 32ºC with an average temperature of 14ºC.

In Israel temperatures for the growing season (April – October) of 2010 ranged from 16^{o}C to 47^{o}C with an average temperature of 32^{o}C, from 14^{o}C to 45^{o}C with an average temperature of 30^{o}C for 2011 and for 2012 ranged from 13^{o}C to 46^{o}C with an average of 31^{o}C (Weather Underground).

4.4.5. *Symptom Expression*

Definitive differences in the symptoms of disease caused by *Dickeya* spp. and *Pectobacterium atrosepticum* is unclear and likely to depend on not only species but the particular isolate; in addition to environmental conditions and even the cultivar involved (Laurila *et al*., 2010; Toth *et al.*, 2011). It is generally assumed that under warm, wet conditions, symptoms of *D. solani* manifest as stem rot but when the weather is cooler and drier, less rotting occurs and symptoms of wilting and hollowing of the stem are common (Lumb *et al*., 1986; Palacio-Bielsa *et al*., 2006; Tsror *et al*., 2009; Toth *et al*., 2011). In many European countries, detection of *Pectobacterium* and *Dickeya* sp. is through visual inspections of the growing crops and distinguishing between symptoms produced by the two genera is difficult (Toth *et al.*, 2011). From our studies, relying on the presence of symptoms would result in infection being missed as in 2010, only 4 out of the 11 (37%) progeny tubers positive for *D. solani* expressed visual symptoms in the plant. In 2011 this number was slightly higher with 8 out of 17 (47%) of the infected progeny tubers expressing plant symptoms; however, in 2012, symptoms were expressed by both plants grown from infected seed found to be positive for *D. solani*. In the cultivar Hermes four out of the ten plants (40%) that were positive for *D. solani* grown from inoculated tubers expressed symptoms.

4.4.6 *Conclusion from Study*

From this limited study, not all inoculated cv. Nicola tubers produced infected daughter tubers, nor did they consistently cause infection in neighbouring plants and tubers. The highest proportion of infected progeny plants was found in 2011, when 47.2% of inoculated seed tubers produced infected progeny plants. The least proportion was found in 2012, when only 5.6% of progeny plants from inoculated seed were found to be infected with *D. solani*. Over the three years, 30 progeny cv. Nicola plants were found positive for *D. solani* from 108 inoculated seed tubers, or 27.8% of progeny tubers grown from inoculated seed were positive for *D. solani*, and only 5 progeny cv. Nicola tubers found positive with *D. solani* were grown from the 432 uninoculated seed tubers (1.6%). This would suggest that transmission, either from infected mother tubers

to progeny tubers and between infected and healthy plants, is inefficient though it is telling to note that the year which showed the highest levels of transmission: 2011, was also the year that experienced a maximum temperature of 25.7^{o}C.

Changes to this study were limited due to the quarantine status of *D. solani* in Scotland (The Seed Potatoes (Scotland) Amendment Regulations 2010). This restricted the study to growing the plants in raised beds in the quarantine section at SASA. Limitations were placed on the soil conditions due to these regulations which may have impacted the transmission of the pathogen. Although *D. solani* has not been shown to survive for a long time in soil, the presence of other micro-organisms could influence its success; for example, in 2012, the observational study in Fera was cut short due to exceptionally wet conditions and the high incidence of late blight amongst the crop (Czajkowski *et al.*, 2010). Whilst current Scottish growing conditions do not seem to facilitate the spread of *D. solani*, global warming and the overall increase in temperatures world-wide could result in *D. solani* remaining as a persistent threat to the Scottish potato industry, especially when considering the potential for latent *D. solani* infection and the potential presence of the pathogen within seed tubers not displaying any disease symptoms (Toth *et al.*, 2015).

Chapter 5. The Survival of *Dickeya solani* on Materials Commonly used in Potato Production and the Susceptibility of *Dickeya solani* to Common Disinfectants

5.1. Introduction

5.1.1 *Spread and Control of* Dickeya solani *in Seed Tubers*

Pectobacterium and *Dickeya* spp. cause disease in a wide range of host species, causing blackleg and tuber soft rot in potato, which makes them among the most important of potato bacterial pathogens (van der Wolf and De Boer, 2007). As they are considered to be seed tuber pathogens, their control has primarily been through seed tuber classification systems within the European Community (Pérombelon, 1974; Toth *et al*., 2011). These classification systems are the responsibility of national jurisdiction and are guided by standardised protocols for the certification of plant materials from the European Plant Protection Organization (Phytosanitary Directive 2000/29/EG). Although the certification schemes have become the accepted approach to control the spread of disease, their success varies and can be dependent on a number of factors related to the growth of the seed crop, including the weather, which limits the detection of latent infections in progeny tubers (Czajkowski *et al.*, 2011). Potato tuber soft rot can occur at any stage of potato production but harvesting and grading are considered the most important stages for cross contamination (Pérombelon & van der Wolf, 2002). Similarly, in storage, infection by *Dickeya* and *Pectobacterium* spp. can lead to rotting of the tubers which can release fluids from the rotting tubers to other neighbouring tubers, thus spreading the infection to healthy tubers (Czajkowski *et al*., 2011).

5.1.2 *Spread and Control of Plant Pathogenic Bacteria after Planting*

Studies of blackleg disease in Scotland have shown that contamination of initially bacteria-free potato stocks occur over three years of growing in the field and contamination was related to the timing of the mechanical handling at harvest and grading in the store, which suggests that the initial contamination came from contaminated machinery (Pérombelon *et al.*, 1980; Czajkowski *et al.*, 2011). Spread of *Dickeya solani* after harvesting occurs due to the rotting tuber breaking down, which can be assisted by physical damage during handling, which releases bacteria onto the machinery and allows the bacteria to spread to subsequent harvested crops which might otherwise have been healthy (Elphinstone & Pérombelon, 1986; Pérombelon & Van der

Wolf, 2002). Disinfection of machinery and equipment used in harvesting and grading can prevent the spread of bacteria between seed lots (Toth *et al.*, 2011). The use of disinfectants on seed tubers to eradicate bacterial pathogens on potato has been suggested; but, many of these will only remove inoculum superficially present on tubers and not within the tuber vascular tissue or inside growing plants (Czajkowski *et al.*, 2013). Studies by Czajkowski *et al.* (2013) looked at whether chemical disinfectants were able to reduce the superficial contamination of potato tubers by *D. solani*; however, he focused on treating seed tubers prior to planting and studying whether symptoms developed in the potato plant. Chemicals are rarely used directly on plants or planting material as they many pose a risk to human and/or animal health and may produce phytotoxic effects (Bloomfield & Scott, 1997; Buck *et al.*, 2003; Celar *et al*, 2007).

Studies using *Pectobacterium atrosepticum* have shown that good sanitation practices between seed lots reduces the spread of *P. atrosepticum* from seed lots with a high incidence to one with a lower incidence (Charkowski, 2015). Once the disease has appeared in the crop it is very difficult to eradicate as methods such as antibiotics and chemical treatments can be difficult on such large scales in addition to the negative side effects they can cause (Czajkowski *et al.*, 2013). Sanitation is therefore essential to prevent the spread of *Dickeya solani* and reduce its incidence.

5.1.3 *Importance of Good Hygiene Practise in Preventing the Spread of Plant Bacterial Pathogens*

Freedom from plant pathogens is not a static occurrence but has to been continuously monitored and maintained during all stages of production, storage and distribution (Janse and Wenneker, 2002). No single strategy is successful in controlling the spread of *Dickeya solani*, therefore, multiple strategies must be implemented including sampling, testing and hygiene based on studies carried out on *Erwinia chrysanthemi* (Janse and Wenneker, 2002). Principles for the control of bacterial pathogens are based on those suggested by Robert Koch in the late 19th century to eradicate cholera; one of these principles implements appropriate measures on contaminated fields and premises and imposing hygienic protocols (Janse and Wenneker, 2002). Successful eradication has been seen with *Ralstonia solanacearum* which causes bacterial brown rot in potato and has been the basis of many of the control measures when *Dickeya solani* was discovered in Scotland in 2009 and they have formed the basis of a number of studies of

the epidemiology of *D. solani* (Janse and Wenneker, 2002). In controlling *R. solanacearum*, measures such as prohibiting the use of irrigation water from the environment, removing contaminated fields from potato production and the disinfection of premises and fields were implemented (Olson, 1976; Persson, 1998). The spread of bacteria was facilitated by the release from infected plant tissue during handling, harvesting, and subsequent planting operations resulting in contamination of storage surfaces, machinery and clothing (Nelson, 1980). The understanding of the ability of *R. solanacearum* to survive on contaminated surfaces under different conditions allowed for better understanding of decontamination and prevention of the spread of the bacteria to uninfected seed (Nelson, 1980; Janse and Wenneker, 2002). The most efficient control of bacterial diseases can be expected through a combination of the use of healthy/tested planting material and good cultivation practices, including strict crop and storage hygiene (Janse and Wenneker, 2002). The importance of hygiene, education and early detection/immediate action is high but is often underestimated (Janse and Wenneker, 2002).

5.1.4 *Specific Aims and Purpose of this Study*

This study attempted to gain more understanding of the persistence and spread of *Dickeya solani* through evaluating the survival of *D. solani* on materials commonly used in potato grading and storage. Five materials: aluminium, hessian, rubber, steel and wood, were used to test whether a strain of *D. solani* could survive, in comparison to *D. dianthicola* and *Pectobacterium atrosepticum*. In addition, ten disinfectants commonly used in agriculture or the laboratory were tested for their ability to control the growth of *D. solani*, in comparison to *D. dianthicola* and *P. atrosepticum*. The Scottish Government, in partnership with Fera, provide guidelines to potato producers to prevent the spread of *Dickeya* spp. In Scotland guidelines include harvesting the infected crop and either selling as ware or complete destruction of the crop. All machinery and material such as equipment used in planting, harvesting, grading and other equipment that has been in contact with the contaminated stock, must be cleaned and disinfected. This study will enable better advice to be provided to producers and the implementation of more appropriate control strategies.

5.2. Materials and Methods: Survival of *Dickeya solani* on Common Materials used in Potato Production

5.2.1 *Materials assessed for survival of* Dickeya *and* Pectobacterium *spp.*

Five materials were used to test whether *Dickeya solani* could survive on common materials used in potato production in comparison to *D. dianthicola* and *Pectobacterium atrosepticum*. These materials included:

1. Rubber from rollers used in grading
2. Aluminium
3. Steel
4. Wood
5. Hessian bag

The aluminium and steel were procured from B&Q, Hermiston Gait, Edinburgh. The aluminium was FFA Concept Anodised Aluminium Flat, measuring 1000mm x 25mm x 2mm. The steel, FFA Concept Varnished Drawn Steel Flat, measuring 1000mm x 16mm x 2mm. The other materials were gathered from the farm at SASA. All samples were cut to an approximate size of 4cm^2 and sterilised by autoclaving at 121^{o}C for 15 minutes.

5.2.2 *Bacterial Suspensions*

Dickeya solani was re-isolated from freezerbeads as previously described in Appendix 2 and grown overnight on Nutrient Agar. The strain selected to represent *D. solani* was isolate MK13, for *D. dianthicola* it was isolate PRI2260 and for *Pectobacterium atrosepticum* it was NCPPB549. MK13 and PRI2260 were grown at 36^{o}C and NCPPB549 grown at 25^{o}C. Bacterial suspensions of 10^8 CFU.ml^{-1} were made by picking colonies from the plates and resuspending in approximately 50 ml of sterile distilled water to give an optical density of 0.8 at 560nm.

5.2.3 *Exposure of Materials and Isolation Methods*

Exposure of the materials to the pathogens was carried out by placing the materials in liquid suspensions and incubating for 48 hours at either 36^{o}C for MK13 and PRI2260 suspensions and 25^{o}C for NCPPB549. After incubation, the materials were removed and left to dry overnight at room temperature in a sterile Petri dish.

Four different isolation procedures were applied in order to recover the test organisms from each material:

Isolation Method One: After drying overnight, the material was placed directly onto CVPM and incubated at either 36ºC or 25ºC for the appropriate sample to see whether the pathogen could be revived from the material. After 48 hours, the plate was checked for the presence of pit-forming colonies.

Isolation Method Two: Once dry, the material was placed in a Falcon tube filled with approximately 50ml of PEM and incubated at 36ºC for the *Dickeya* spp. and 25ºC for *Pectobacterium atrosepticum* for 48 hours. After 48 hours, the storage material was removed and placed directly on CVPM agar and incubated at either 36ºC for *Dickeya* spp. and 25ºC for *P. atrosepticum* for 48 hours to assess the development of any pit-forming colonies. One hundred microliters of the used PEM suspension was plated onto CVPM agar and incubated for 48 hours at either 36ºC or 25ºC and pit-forming colonies counted.

Isolation Method Three: The dry contaminated storage material was rinsed with 1ml of sterile water, which was collected. The rinsed storage material was placed onto CVPM as incubated for 48 hours at 36ºC for MK13 and PRI2260 and 25ºC for NCPPB549. In addition, 100µl of the collected rinse water was spread onto CVPM agar and also incubated as described for the storage material.

Isolation Method Four: After drying the storage material was rinsed with 1ml sterile distilled water and isolated as described in method three; however, the storage material was then placed into PEM, rather than directly onto CVPM agar, and isolation carried out as described in Method Two. Formation of pit-forming colonies was then assessed.

5.2.4 *Exposure of Materials and Isolation Revised Methods*

Initial results from the method described in Chapter 5.2.3. suggests that the pathogens did not survive on the storage materials as no pit-forming colonies were found from any of the isolation methods. The original method was then modified slightly. MK13, PRI2260 and NCPPB549 were used again as the pathogens and re-isolated as previously described above. Bacterial suspensions of 10^8 CFU.ml^{-1} were made by picking colonies from the plates but instead of adding to sterile distilled water, the

colonies were added to approximately 50ml of PEM to give an optical density of 0.8 at 560 nm.

Exposure of the materials was repeated as above by placing the materials in the PEM suspension and incubating for 48 hours at either 36ºC for MK13 and PRI2260 and 25ºC for NCPPB549. After incubation, the materials were removed and left to dry for 4 hours at room temperature.

Three different isolation procedures were applied in order to recover the test organisms from each material:

Isolation Method Five: After drying, the material was placed directly onto CVPM and incubated at either 36ºC or 25ºC for the appropriate pathogen to see whether the pathogen could be isolated from the material. After 48 hours, the plate was checked for the presence of pit-forming colonies.

Isolation Method Six: Once dry, the material was placed in a Falcon tube filled with approximately 50ml of PEM and incubated at 36ºC for the *Dickeya* spp. and 25ºC for *Pectobacterium atrosepticum* for 48 hours. After 48 hours, the storage material was removed and placed directly on CVPM and incubated at either 36ºC for MK13 and PRI2260 and 25ºC for NCPPB549 for 48 hours to assess for the development of any pit-forming colonies. One hundred microliters of the used PEM suspension was plated onto CVPM and incubated for 48 hours at either 36ºC or 25ºC and pit-forming colonies counted.

Isolation Method Seven: The dry infected storage material was rinsed with 1ml of sterile water, which was collected. The rinsed storage material was placed onto CVPM as incubated for 48 hours at 36ºC for MK13 and PRI2260 and 25ºC for NCPPB549. In addition, 100µl of the collected rinse water was spread onto CVPM and also incubated as described for the storage material.

5.3 Materials and Methods: Susceptibility of *Dickeya solani* to Disinfectants Commonly Used in Agriculture

The effectiveness of a number of disinfectants, as listed in Table 5.1, commonly used in agriculture were tested against *Dickeya solani* and compared to the sensitivity of *D. dianthicola* and *Pectobacterium atrosepticum*.

5.3.1 *Growing Strains to Test Against Disinfectants*

Dickeya solani was re-isolated from freezerbeads as described in Appendix 2 and grown overnight on Nutrient Agar. The strain selected to represent *D. solani* was isolate PRI2222, *D. dianthicola* isolate PRI2260 and *Pectobacterium atrosepticum* NCPPB549. PRI2222 and PRI2260 were grown at 36ºC and NCPPB549 at 25ºC. Bacterial suspensions of 10^8 CFU.ml^{-1} were made by picking colonies from the plates and re-suspending in approximately 50ml of sterile distilled water to give an optical density of 0.8 at 560 nm.

5.3.2 *Susceptibility Testing Against Disinfectants*

A range of disinfectant concentrations were tested. Initially these were:

1. Lowest active range (LAR): the manufacturer's recommended dilution for general use
2. 50% dilution of LAR concentration
3. 25% dilution of LAR concentration

Table 5.1 Details of Disinfectants and their Dilutions Used in Sensitivity Testing of *D. solani.*

Product	Manufacturer	Active ingredient	Recommended dilution for general use
Fam30	Evans Vanodine	Iodophor	1:180
GPC8	Evans Vanodine	Glutaraldehyde	1:35
Halamid	Axcentive SARL	Chloramine-T	0.3:100
Jet5	Certis	Peroxyacetic acid	1:250
Jeyes Fluid	Jeyes	Cresol	1:20
Mikrozid	Schülke	Ethanol	Not applicable – apply undiluted
Sodium hypochlorite	Enov	Sodium hypochlorite	Not applicable – apply undiluted
V18	Evans Vanodine	Iodophor	1:500
Vanoquat	Evans Vanodine	Quaternary Ammonium	1:300
Virkon S	LANXESS	Oxone (potassium peroxymonosulfate	1:100

In each case the disinfectants were tested against the three test organisms by adding 100 µl of the bacterial suspension described above to 900 µl of the disinfectant solution. The mixture was vortexed and left for the required incubation time of either 5, 10 or 30 minutes at room temperature. Prior to the end of each contact time period, the suspension was vortexed again. The suspension was then centrifuged at 13,000 rpm for one minute and the disinfectant (the supernatant) discarded. The pellet was re-suspended in 1ml of sterile distilled water (SDW) and centrifuged again for one minute at 13,000 rpm, again the supernatant (SDW) was discarded. The pellet was finally re-suspended in 1 ml of SDW and 100 µl of the suspension plated onto CVPM agar plates and incubated for 48 hours at 36^{o}C for PRI2222 and PRI2260 and 25^{o}C for NCPPB549. The number of pit-forming colonies was assessed after incubation for 48 hours.

Initial results showed no growth at the LAR, 50% LAR and 25% LAR for any of the disinfectants tested in Table 5.1. The experiment was repeated using more dilute disinfectant solutions, specifically 10% LAR, 5% LAR and 1% LAR. These also

showed no growth for any of the disinfectants from Table 5.1 so a further experiment using dilutions of 0.5%, 0.4%, 0.3%, 0.2% and 0.1% LAR was carried out.

After colony counts were completed, pit-forming colonies were re-isolated onto Nutrient Agar and incubated for 24 hours at 36^{o}C for PRI2222 and PRI2260 and at 25^{o}C for NCPPB549. Single colonies from overnight Nutrient Agar plates were picked off using a sterile loop and mixed into 1ml sterile distilled water. The suspension was boiled at 100^{o}C for 5 minutes and stored at -20^{o}C prior to use in the Nassar PCR Assay (Nassar *et al.*, 1996) and the real-time PCR Assays as described in Chapter 2 to confirm the survival of *Dickeya* spp. after exposure to the disinfectants. *Pectobacterium atrosepticum* identification was assumed by the growth of pit-forming colonies on CVPM agar plates at 25^{o}C and a negative result using the Nassar PCR Assay (Nassar *et al.*, 1996).

5.4 Results of Survival of *Dickeya solani, D. dianthicola* and *Pectobacterium atrosepticum* on Materials Commonly Used in Potato Production.

The initial results using methods one through four showed no growth of any of the three pathogens from any of the storage materials which would suggest a loss of the pathogens during the incubation process. In attempt to resolve the viability of the pathogens, the experimental methods were revised and outlined in methods five through seven.

In the revised methods, the bacteria were grown in pectate enrichment media (PEM) rather than sterile distilled water and applied to the materials in PEM. The PEM provided a nutrient source for all three pathogens and is commonly used to increase the bacterial load when testing environmental samples showing blackleg/soft rot symptoms. All three pathogens were able to persist and be grown from all five storage materials, as outlined in Table 5.2. Using isolation method 5, a single pit-forming lawn formed surrounding the infected storage material. The lawn was also formed in subsequent isolation methods 6 and 7 and in both additional methods, pit-forming colonies were produced from the PEM and the rinse water used however these were too numerous to count (TNTC).

Subsequent experiments attempted to use mashed up potato material as an alternative media instead of PEM; however, *Dickeya solani* was unable to survive in the potato material and on any storage material exposed to the cultures. The potato material provided a more appropriate environmental source of nutrients as the pathogens typically break down the pectate in the potato tuber. There has been speculation soft rot erwiniae can spread through contaminated plant material on materials used in potato production but, the pathogens did not survive, or at least could not be recovered, from potato material and storage material in this study.

Table 5.2 Revival of Pathogens from Storage Materials Incubated in Spiked PEM

Storage Material	Pathogen	Isolation Method 5*	Isolation Method 6*		Isolation Method 7*	
		Storage material on CVPM	Storage material CVPM	PEM on CVPM	Rinse water on CVPM	Storage material on CVPM
Aluminium	*Dickeya solani*	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
	D. dianthicola	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
	Pectobacterium atrosepticum	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
Hessian	*D. solani*	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
	D. dianthicola	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
	P. atrosepticum	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
Rubber	*D. solani*	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
	D. dianthicola	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
	P. atrosepticum	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
Steel	*D. solani*	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
	D. dianthicola	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
	P. atrosepticum	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
Wood	*D. solani*	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
	D. dianthicola	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn
	P. atrosepticum	Pit-forming lawn	Pit-forming lawn	TNTC	TNTC	Pit-forming lawn

TNTC – Too numerous to count

*Isolation method described in section 5.2.4

5.5 Results of the Susceptibility of *Dickeya solani* to Disinfectants Commonly Used in Agriculture

In all ten different disinfectants were tested against the three species studied here; *Dickeya dianthicola*, *D. solani* and *P. atrosepticum*. All products studied were selected either because they were recommended for use in agriculture or were freely available.

The results in Table 5.3 show that if the disinfectants included in the study are applied at the concentrations recommended by the manufacturer for general use, even at the shortest contact time they are effective at controlling all three species, as no growth was observed on the CVPM. The effectiveness of each disinfectant varied as the concentrations were diluted to 0.5, 0.4, 0.3, 0.2 and 0.1% of the recommended dosage. Sodium hypochlorite and Vanoquat were the only disinfectants that showed effectiveness against all three pathogens even to 0.1% dilution of the recommended general use dilution and at the shortest contact time.

Fam30 was effective at an exposure time of five minutes for *Dickeya solani* down to a dilution of 0.3% of the recommended concentration with 5.1×10^{-1} CFU.ml^{-1} seen at 0.2% dilution. After ten minutes, colony numbers of 1.04×10^{-1} CFU.ml^{-1} were seen at 0.2% and growth at 0.1% and after 30 minutes, growth was only seen at 0.1%. For *Pectobacterium atrosepticum*, growth was seen after five minutes at 0.2% and 0.1%. After ten minutes, growth was seen at 0.3, 0.2 and 0.1% and after 30 minutes growth was seen at 0.2% and 0.1%. Fam30 was completely effective against *D. dianthicola* regardless of concentration or contact time

GPC8 was effective against *Dickeya solani* after an exposure of five minutes down to 0.5%; however a small amount of growth was seen at 0.4 and 0.3% with colony counts of two and one respectively and uncountable growth at 0.2 and 0.1%. After exposure of ten and 30 minutes no growth of *D. solani* was seen. When *Pectobacterium atrosepticum* was exposed to GPC8 for five minutes at dilutions of 0.2% and 0.1% colony growth was seen (2 and 231 total colonies respectively). For all other exposure times and dilutions, no growth was seen. GPC8 was effective against *D. dianthicola* at all exposure times and dilutions.

Halamid was effective at exposure times of 10 and 30 minutes for all three pathogens; however, a small number of *Dickeya solani* colonies (ranging from 0-3) was seen when exposure was limited to five minutes for dilutions 0.5, 0.4, 0.3, 0.2 and 0.1%.

Jet5 was not effective against *Dickeya solani* growth after five minutes of exposure and growth was seen at all dilution levels. After ten minutes exposure, growth was seen at all dilution levels with colony counts of 1.56×10^{-1} CFU.ml^{-1} at 0.5% and too many colonies to count were observed at the other dilution levels. Colony counts of $7x10^{-1}$ CFU.ml^{-1} were seen at a dilution of 0.4% and an exposure time of 30 minutes and too many to count at 0.5%; for the other dilution levels no growth was seen. *Pectobacterium atrosepticum* was not sensitive to Jet5 at 0.1% dilution and exposure of five minutes and one colony was present. After ten minutes exposure, no growth was found at 0.5%; however four colonies were counted at 0.4% and too many for quantification were seen at dilutions of 0.3, 0.2 and 0.1%. Thirty minutes of exposure showed sensitivity of *P. atrosepticum* at dilutions of 0.5% and 0.4% but growth at 0.3% (a single colony), 0.2% (16 colonies) and 0.1% (too many to count). Jet5 was effective against *D. dianthicola* under all conditions.

Although Jeyes fluid provided effective control when using the manufacturer's recommended concentration at 0.5% and below it was ineffective at all exposure times.

Mikrozid AF and V18 were ineffective against *Dickeya solani* and *Pectobacterium atrosepticum* at all dilutions and exposure times. Both were effective against *D. dianthicola* at all dilutions even at the shortest contact time.

Virkon S was only effective against *Dickeya solani* at an exposure period of 30 minutes and against *D. dianthicola* for all conditions except 0.1% dilution and exposure of 10 minutes when one colony grew. *Pectobacterium atrosepticum* was sensitive to Virkon S under all conditions.

Table 5.3 Susceptibility of *Dickeya solani*, *D. dianthicola* and *Pectobacterium atrosepticum* to a Range of Disinfectants

Commercial Product (General use dilution)	**Strains**	**Contact times (min)**	**Colony Count***					
			%					
			100	**0.5**	**0.4**	**0.3**	**0.2**	**0.1**
FAM 30 (1:180)	*D. solani*	5	0	0	0	0	510	TNTC†
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	TNTC	TNTC
	D. solani	10	0	0	0	0	104	TNTC
	D. dianthicola	10	0	0	0	0	0	1
	P. atrosepticum	10	0	0	0	TNTC	TNTC	TNTC
	D. solani	30	0	0	0	0	0	TNTC
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	TNTC	TNTC
GPC8 (1:35)	*D. solani*	5	0	0	2	1	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	2	231
	D. solani	10	0	0	0	0	0	0
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0

	Strains	Contact times (min)	Colony count*					
			%					
			100	0.5	0.4	0.3	0.2	0.1
	P. atrosepticum	30	0	0	0	0	0	0
Halamid (03:100)	*D. solani*	5	0	1	2	0	1	3
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	0
	D. solani	10	0	0	0	0	0	0
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0
Jet 5 (1:250)	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	1
	D. solani	10	0	156	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	4	TNTC	TNTC	TNTC
	D. solani	30	0	0	0	0	70	TNTC
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	1	16	TNTC
Jeyes Fluid (1:20)	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	5	0	TNTC	TNTC	TNTC	TNTC	TNTC

	Strains	Contact times (min)	Colony Count* %					
			100	**0.5**	**0.4**	**0.3**	**0.2**	**0.1**
	D. solani	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
Mikrozid AF liquid	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
Sodium hypochlorite (14:100)	*D. solani*	5	0	0	0	0	0	0
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	0
	D. solani	10	0	0	0	0	0	0
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0

	Strains	Contact times (min)	Colony count*					
			%					
			100	0.5	0.4	0.3	0.2	0.1
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0
V18 (1:500)	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	TNTC	TNTC	TNTC	TNTC	TNTC
Vanoquat (1:300)	*D. solani*	5	0	0	0	0	0	0
	D. dianthicola	5	0	0	0	0	0	0
	P. atrosepticum	5	0	0	0	0	0	0
	D. solani	10	0	0	0	0	0	0
	D. dianthicola	10	0	0	0	0	0	0
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0
Virkon S (1:100)	*D. solani*	5	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	0	0	0	0	0	0

	Strains	Contact times (min)	Colony Count*					
			%					
			100	0.5	0.4	0.3	0.2	0.1
	P. atrosepticum	5	0	0	0	0	0	0
	D. solani	10	0	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	0	0	0	0	0	1
	P. atrosepticum	10	0	0	0	0	0	0
	D. solani	30	0	0	0	0	0	0
	D. dianthicola	30	0	0	0	0	0	0
	P. atrosepticum	30	0	0	0	0	0	0
-ve control (water)	*D. solani*	5	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	5	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	5	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	10	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	10	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	10	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. solani	30	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	D. dianthicola	30	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	P. atrosepticum	30	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

*Colony count given in CFU.ml^{-1}. †, TNTC- colonies too numerous to count

5.6 Discussion

Measures implemented to control the spread of bacterial potato pathogens are multi-faceted and rely on the production of seed that are tested to be free of the pathogens, measures to reduce the amount of inoculum that may already be present and good agronomic practices (van der Wolf and De Boer, 2007). The success of the control and eradication of bacterial diseases after their introduction to new areas largely depends on the available infrastructure to facilitate their detection and subsequent removal from the environment. Disease control is difficult and relies mainly on preventative measures such as avoiding plant injuries and contamination during harvesting and handling. In areas where *Dickeya* spp. are not present, measures should concentrate on preventing the introduction of the pathogen (Toth *et al*., 2011). Infected seed tubers present the most likely source of pathogen introduction and almost all new findings of *D. dianthicola* and *D. solani* can be traced back to the seed source and when it was introduced. Once it has been introduced, measures must be developed to stop the pathogen's spread between crops once it has been introduced and it has been suggested to avoid mechanical harvesting during the early phases of pre-basic seed tuber multiplication as this may reduce the rate of contamination of healthy stocks and the spread of *Dickeya* spp. (Czajkowski *et al.*, 2011).

Guidelines to growers set by the Scottish Government state that grading is a key stage for preventing disease spread, as the machinery can become contaminated by one crop and pass the disease to crops which are graded later (SASA). In the Code of Practice for the Management of Agricultural and Horticultural Waste (Fera, 2008), it is advised that producers use a "proprietary disinfectant suitable for the organism(s) potentially present and follow all the manufacturer's label recommendations." Based on this study, it is clear that all ten disinfectants tested, which are commonly available and used in both an agriculture and laboratory setting, are effective at controlling *Dickeya solani*, *D. dianthicola* and *P. atrosepticum*, especially when applied at the manufacturer's recommended dosage for general use. When diluted to concentrations of 0.5, 0.4, 0.3, 0.2 and 0.1% of the manufacturer's recommended dose for general use the effectiveness varied and from the results it is clear that the only disinfectants effective against all three pathogens were sodium hypochlorite and Vanoquat even at dilutions as low as 0.1% of the recommended dose for general use.

This study has shown that there is a wide variety of products available to control the spread of *Dickeya solani*, in addition to *D. dianthicola* and *Pectobacterium atrosepticum*, and if used properly and according to the manufacturer's recommended concentration for general use, that they can control the spread of *D. solani*. Whilst sodium hypochloride and Vanoquat are effective even at dilutions lower than the recommended dosage, others such as Jeyes Fluid are not effective when diluted, not only for *D. solani* but also for *D. dianthicola* and *Pectobacterium atrosepticum*., Any differences between the effectiveness of disinfectants were only seen at very low concentrations of those recommended for general use. In order to control the spread of blackleg and tuber soft rot, several concepts have been considered, and whilst there have been varying rates of success, those based on preventing contamination have been the most successful and alongside seed certification schemes are the most widely used (Czajkowski *et al.*, 2011). These findings suggest that the spread of *D. solani* by contaminated machinery can be prevented if the machinery is kept clean through the use of an appropriate disinfectant, applied according to the manufacturer's instructions and the chances of transmission from an infected seed lot to a healthy lot could be greatly reduced.

This study found that *Dickeya solani* does not easily persist on common surfaces used in potato production such as wood, steel, rubber, hessian and aluminium unless a nutrient source was provided, as in the form of pectate enrichment media evaluated in this study. When macerated potato was used as nutrient source there was no survival of any of the three pathogens. Based on this study, it is unlikely that surfaces previously exposed to *D. solani* contamination would provide a source of further *D. solani* spread. This is in contrast to previous studies, including those carried out in Fera as part of this project looking at commercial crops in England which found *D. solani* populations on harvested tubers remained constant during storage for six months in controlled environments at 8^{o}C and populations increased when stored at on-farm temperatures of between 8-12^{o}C; other studies have demonstrated that tuber to tuber contact with rotting, infected tubers poses a major threat for disease spread (J. Elphinstone, Fera, unpublished data).

Previous studies on blackleg-causing pathogens have shown internal contamination within the vascular system, in tuber lenticels and also on the tuber periderm in wounds that were created during handling and through this the bacteria can exist for long periods of time (Czajkowski *et al.*, 2010b; de Boer, 2002; Elphinstone and Pérombelon

1986). Wounds can become contaminated with bacteria by smearing from rotting tubers during post-harvest handling and can play an important role in disease transmission from one generation to the next which has had devastating effect in storage and caused the loss of entire crops through the spread of disease in storage (Pérombelon, 1976).

Previous monitoring of seed contamination in five different Scottish seed producing farms of crops derived from stem cuttings over a five year period demonstrated that farms which regularly applied good hygiene measures such as washing and disinfection of machines used at various stages of seed production produced cleaner seed than others (Pérombelon *et al.*, 1980). Therefore, good hygiene measures can be assumed to reduce the risk of introducing soft rot/blackleg pathogens in pathogen-free crops (Pérombelon and Kelman, 1980; Pérombelon, 2002). Good storage management can also prevent tuber decay and reduce the amount of pathogen within the lot, thereby reducing disease risk (Czajkowski *et al.*, 2011).

Using pathogen-free seed in combination with good hygiene and cultivation practices are the most important management tools as it is possible for *D. solani* to spread through a potato crop at all points of potato production. Although in this study, *D. solani* was not found to survive directly on materials commonly used in potato production, it is possible that the pathogen could survive if plant debris was present (van der Wolf *et al.*, 2007). Contamination can occur from machinery during planting, during spraying which can spread the bacteria to haulms, roots and tubers, during flailing, harvesting and sorting and washing but disinfection can substantially reduce the spread of bacteria. This study shows that practicing good hygiene and cleanliness could prevent the spread of the pathogens as the ten disinfectants tested are easily accessible and when used according to manufacturer's instructions are effective against *D. solani*, *D. dianthicola* and *P. atrosepticum*.

Chapter 6. General Discussion and Conclusions from the Experimental Studies

6.1 The Importance of the Research

Dickeya spp. are the primary pathogens causing soft rot and blackleg diseases of potato (Sławiak *et al.*, 2009; Toth *et al*., 2011). Until 2000, the dominant cause of blackleg and tuber soft rot in potato was considered to be *Pectobacterium atrosepticum*, with *Dickeya* spp. considered more problematic at higher temperatures and humidity, such as in tropical and subtropical regions and causing disease in ornamentals or crops grown in glasshouses (Pérombelon, 2002). The *Dickeya* sp. associated with potato blackleg in Europe had previously been exclusively *D. dianthicola*, as strains were better adapted to the climate conditions in Europe due to their lower growth temperature (Janse & Ruissen, 1988). In 2005 *D. solani* emerged, leading to a rise in blackleg and soft rot incidences in potato in Europe and since its emergence has spread rapidly throughout the continent, having been isolated in many European countries and extending Israel and Georgia (Tsror *et al*, 2011; Sławiak *et al.*, 2009; van der Wolf *et al*., 2014). On potato, *Dickeya solani* appears to be more virulent than *P. atrosepticum* and the previously isolated *Dickeya* spp. (Czajkowski *et al.*, 2012).

Disease caused by *Dickeya solani* can manifest at any point during plant growth, harvest or even during tuber storage or during transportation, especially when environmental conditions are favourable. Symptoms can include blackleg, aerial stem rot, chlorosis, and wilting in addition to rotting of either pre-emergence seed tubers in the field or soft rot of the tubers in storage (Pérombelon, 2002). Disease caused by *D. solani* appears to be more severe than those caused by other *Dickeya* species and also than that of *Pectobacterium atrosepticum*, which is a more established potato pathogen, regardless of environmental conditions (Toth *et al.*, 2011; Czajkowski *et al*., 2012; van der Wolf *et al.*, 2014). During the growing season, high temperatures have been suggested as a driving factor in determining which bacterial species dominates in causing disease (Luck *et al.* 2011). Plant pathogenic bacteria respond to variations in environmental conditions in order to facilitate pathogenesis. To be successful, a pathogen must establish symptomatic infection in the host and temperature has been shown to signal the expression of virulence factors during infection (Czajkowski *et al*., 2016). During the potato growing season, temperature fluctuations are common,

therefore it is be advantageous for pathogenicity of plant pathogens to be expressed over a wider range of environmental conditions (Czajkowski *et al*., 2016*)*.

Dickeya solani emerged in 2005/2006, most likely crossing from horticultural crops to potato, as the earliest known strains of *D. solani* were isolated from hyacinth and strains of *D. solani* continue to be isolated from this crop (Parkinson *et al.*, 2009; Chen *et al.*, 2015). Within the European Community, *D. solani* is a regulated, non-quarantine pest and is controlled by the majority of countries through the use of potato seed certification schemes. Through these schemes, potato seed stock is generally initiated from pathogen-tested nuclear stock microplants and field production is limited to a restricted number of generations to avoid the build-up of pathogens with each field multiplication (Toth *et al.*, 2011). All seed classification schemes are reliant on visual inspections of field crops and tubers in storage and whilst there is zero tolerance to soft rot and blackleg diseases in high grade material, latent infections exist and can be overlooked in visual inspections, which suggests the use of post-harvest testing to monitor seed stocks for the presence of *D. solani* may be advantageous (Czajkowski *et al.*, 2011). Scotland was the first country in Europe to enforce testing of all non-indigenous seed stocks prior to planting in order to ensure freedom from *D. solani* (Kerr *et al.*, 2010). In addition to a zero tolerance system for *Dickeya* sp. in seed tubers, Scotland employs a visual inspection supported by laboratory testing, in which high-risk crops, i.e., those of non-Scottish origin, are tested in addition to 10% of the indigenous production also being surveyed which confirms freedom from *Dickeya* sp. The movement of latently infected seed is the principal transmission route of *Dickeya* sp. and measures which assure plant seed health are gaining traction in a number of countries. The UK set up the Safe Haven Scheme (https://potatoes.ahdb.org.uk/seed-exports/safe-haven-certification-scheme), which is an industry led initiative that ensures that only disease-free microplants can enter the production chain and that field-grown generations can only be grown on agricultural units that do not handle seed from outside the scheme. Through such schemes, healthy planting material can be passed through the production chain without the possibility of introduction of infection from other sources of seed tubers. The control of *D. solani*, especially in Europe, comes from the use of seed certification schemes, however, it is because of the seed certification schemes that losses due to *D. solani* are seen, as crops are downgraded or unable to be classified due to the presence of disease. As national tolerances for classification vary by country so does the economic impact from *Dickeya* disease. Losses of up to €30 million have been seen in

the Netherlands and downgrading/rejections of up to 30% have been seen in Israel (Prins and Breukers, 2008; Tsror *et al*, 2009).

Once *Dickeya solani* has become established in a crop, there is little which can be done to reduce its impact and to reduce the risk of spreading the disease further. Cleaning and disinfection of machinery, equipment and grading lines are crucial and identifying disinfectants which are effective against *D. solani* is important to prevent the spread of disease.

6.2 Aims of the Research

The aim of this project was to understand the biology and epidemiology of *Dickeya solani* in potato and to that end we aimed to fulfil the following objectives:

1. To refine, validate and apply diagnostic, detection and typing methods specifically for *Dickeya solani* to assist with designing adequate control measures and understand the introduction and evolution of the pathogen.
2. To evaluate the risk of spread to Scottish grown seed potatoes by understanding the transmission of the pathogen from infected tubers under field conditions.
3. To improve the understanding of the transmission and survival of *D. solani* in storage and the susceptibility of *D. solani* to common disinfectants to improve control of the pathogen once introduced to a crop or seed lot.

6.3 Development of a MLSA System

At the beginning of this project, the true classification of *Dickeya solani* was unclear, in particular, the diversity between the different strains of *D. solani* recovered from various geographical locations throughout Europe and Israel, and the relationship between *D. solani* and other members of the genus *Dickeya* and the level of diversity between the strains was unresolved. A number of approaches, each of which were able to clarify the relationship between strains at various levels of resolution, were applied throughout the larger *D. solani* project, however, MLSA was the technique focused on in this component of the project. The use of the MLSA system resulted in appropriate discrimination between isolates of *D. solani* and other *Dickeya* sp. and isolates from closely related genera and enabled the publication of an online database containing the sequences of the housekeeping genes used for the analysis. The creation of the online database, which other researchers are able to access, enables a global mechanism by

which *D. solani* can be characterised and its spread monitored. Analysis with MLSA further suggests that *D. solani* is a clonal pathogen as little variation is seen between the strains regardless of the country of origin or the host. With the inclusion of the addition genes *dnaX* and *dnaJ* in this study, global studies can be better utilised, as previously there was variation between the genes used for analysis within Europe. The MLSA system has already begun to be used as part of the EUPHRESCO project in the identification of *Dickeya* to a species level and shows potential for future use with monitoring and controlling the spread of the pathogen.

Through the identification of SNPs, strains can be distinguished at a higher level of resolution, with the potential to characterise new strains, especially those responsible for new outbreaks, SNP analysis must be applied only when isolates have been identified as *D. solani*, which requires the use of other typing and characterisation methods such as those employed with the MLSA method. Based on the SNP analysis in this research, the majority of *D. solani* isolates were separated into two groups; The separation was not based on geographical isolation or the date of isolation. The SNP analysis did support the data gathered from the MLSA study in that MK10, which was slightly different to the other isolates which formed a homologous clade, did not fall into either group of SNPs. There is potential for further research into the significance of the SNPs as the use of SNP analysis in the future would allow for additional fingerprinting and monitoring of outbreaks. The identification of the *D. solani* SNPs in this project has begun the process of developing a new diagnostic system; however, more work is needed before such techniques can be applied.

6.4 Real-time Diagnostic Test and Typing Methods for *Dickeya solani.*

With the spread of *Dickeya* sp. in potato crops throughout Europe, Scotland introduced a zero-tolerance for all *Dickeya* species through legislation in 2010 (The Seed Potatoes (Scotland) Amendment Regulations 2010). Scotland is unique and most other European countries do not have, and now cannot have, such control measures in place. The spread of *D. solani* is believed to be primarily through the trade in seed potatoes; therefore, control is through prevention of the introduction of disease to any uncontaminated crops. Focusing on Scotland, *Dickeya* spp. have been included in the seed certification scheme to prevent infected seed being planted in Scotland and to ensure that no *Dickeya*- contaminated seed is exported from Scotland.

The mechanism of real-time PCR is similar to that of conventional PCR, which had been the previously accepted method for identification of *Dickeya* spp. in combination with the sequencing of either the *recA* or *dnaX* gene to identify to a species level. Using real-time PCR allows for the amplification of DNA to be monitored in real-time, without the need for post-test visualisation such as through gel electrophoresis. This significantly cuts down on the time taken to obtain a result. With such schemes as the Safe Haven, the potato producers and government alike rely on the appropriate, reliable and speedy identification of any potential disease that might be present. Through this project it has been possible to increase the specificity of the PCR test by assessing two assays that could correctly identify *D. solani* and within a shorter time frame than previous methods. Although it has not been possible to refine the sampling methods for the isolation and identification of bacteria, the PCR methods proposed allows for the application of automated systems such as liquid handling robots which enables high throughput analysis and will reduce the time taken for identification.

The two assays have shown promising results and have the potential to replace the current diagnostic method for *D. solani*. The ring test built upon recommendations by the IPPO for validation of tests for plant pathogens and involved six independent laboratories carrying out an assessment of the two protocols. The assays were also verified by NAK in the Netherlands which had designed their own real-time PCR assays. In particular the assay SOL-C which was designed using the primer pipeline is being used by Fera and in the Netherlands for identification of *D. solani*. Both the fusA and SOL-C primers have been included in the list of preferred primers for ring testing within Europe through a EUPHRESCO *Dickeya* project to standardise testing across Europe. The approach using whole genome comparisons which was incorporated into this project is highly novel and has good potential for rapid diagnostics development for a range of future targets.

6.5 The Transmission of *Dickeya solani* under Scottish Growing Conditions

Due to legislation in Scotland, planting of *Dickeya* infected seed is limited and whilst environmental conditions were as close as possible to real-life, there were limitations in where and how infected seed could be planted, which may have affected the results in this study. Other studies have suggested that growth conditions such as moisture and temperature and the presence of symptoms can all impact the transmission of the disease to daughter tubers. Throughout the three growing seasons of 2010, 2011 and

2012, infection was detected in only a minority of plants and symptoms were not visible in all plants from which progeny tubers tested positive for *Dickeya* infection. From the studies carried out in the Scottish quarantine raised beds, it could be assumed that the growth conditions in Scotland do not support the spread of disease during the growth; however, we cannot discount the potential of pathogen spread during storage or harvest. Overall, there was limited evidence to suggest spread between plants either in the beds or between tubers planted in pots. These results are in contrast to other studies carried out in continental Europe where spread has been seen between plants and from infected mother tubers to the progeny. As the climate in Scotland is cooler than that seen in continental Europe, this may account for the lack of spread of the pathogen in growing plants.

6.6 Susceptibility and Sensitivity to Disinfectants and *Dickeya solani* Survival in Storage

The control of *Dickeya* infection has been through preventing the introduction of the pathogen rather than trying to treat the infection once it has taken root. Adequate husbandry and good standards of cleanliness are crucial for preventing the introduction and subsequent spread of the pathogen. From our studies, it is apparent that when using the recommended manufacturer's concentration, all disinfectants, which are commonly used in agriculture, can prevent the survival of *D. solani* in addition to *D. dianthicola* and *Pectobacterium atrosepticum*. When sub-optimal concentrations were tested, differences in efficacy were seen. Sodium hypochlorite and Vanoquat remained effective against all three species across all concentrations tested. These results suggest that if machinery is kept clean and the correct disinfectants applied at the manufacturer's recommended concentrations, the possibility of spread and subsequent spread from infected crops to healthy ones could be greatly reduced.

The spread of *Dickeya solani* has been assumed to be the same as that for *Pectobacterium atrosepticum* and thus spread is possible through infected machinery and other contaminated surfaces during harvesting, processing and storage., Complementary studies assessing the survival of *D. solani, D. dianthicola* and *Pectobacterium atrosepticum* on materials commonly used in potato harvest and storage including wood, steel, rubber and hessian suggest that *D. solani* does not survive well on these materials. When a nutrient source, in the form of pectate enrichment media, was supplied, *D. solani* survived on some of the materials. When macerated potato

tissue was tested as the nutrient source, there were poor survival rates, which would suggest that spread of *D. solani* is unlikely to occur through its presence on the storage materials. Other similar experiments, such as those carried out at Fera (Elphinstone, unpublished data) as part of this project, suggest that tuber to tuber contact, especially when rotting is present, may facilitate the spread of disease.

6.7 Conclusions

The research in this project supports the identification of *D. solani* as a clonal pathogen and the data has been used to support its classification as a novel species (van der Wolf *et al.*, 2014). MLSA and subsequent SNP analysis hint that diversity may exist and its significance may become clearer as more research is carried out, especially on the spread and pathogenesis of *D. solani*. Through this research project, it has been possible to develop two new PCR-based diagnostics specific for *Dickeya solani* which are now being used and recommended across Europe for identification of *D. solani*. This progress will ensure that *D. solani* testing across Europe and the rest of the world is consistent. The use of MLSA and SNP analysis allow for appropriate discrimination between isolates. As *Dickeya solani* has been identified as a clonal pathogen, the use of such typing methods provides improved discrimination and more appropriate grouping of isolates The use of such typing systems can also provide information and aid with tracking strains and outbreaks. Studying the spread of *D. solani* from infected seed tubers determined that spread was more limited than expected and may be due to the climate and soil moisture conditions in Scotland which differ from those in continental Europe. *Dickeya solani* does not appear to survive well on common materials used in potato production however the potential remains for contamination from rotting to healthy tuber to occur during handling and processing. Taking this into consideration, the use of appropriate disinfectants during production could be an effective way to reduce the potential for pathogen spread, especially when used at concentrations recommended by the manufacturers.

From this project, it can be assumed that *Dickeya solani* is poorly suited to survive under potato growing conditions in Scotland, based on the laboratory conditions tested. There remains a moderate risk to potato production in Scotland; therefore, it is important to continue to control seed imports and recommend appropriate measures in production such as the use of effective disinfectants. There are a number of variables that could account for our results, including the isolates used in our experiments. In our

experiments, historical isolates were utilised which had been stored at -80^{o}C and revived. It is possible that some of the pathogenicity factors of these isolates were lost during the growth of these strains under laboratory conditions. In addition, under the conditions present, there was variation of pathogens which may influence the survival of *D. solani* and its dominance over other strains.

Appendix 1. List of Strains Used in Studies.

Table A.1 List of Strains in the SASA Collection.

Strain	Attributed Species	Host isolated from	Country of isolation	Date of isolation
MK1	*Duc3**	River 1	Scotland	23/07/07
MK2	*Duc3*	River 1	Scotland	23/07/07
MK3	*Duc3*	River 1	Scotland	04/07/07
MK4	*Duc3*	River 1	Scotland	04/07/07
MK5	*Duc3*	River 1	Scotland	04/07/07
MK6	*Duc3*	River 1	Scotland	04/07/07
MK7	*Duc3*	River 1	Scotland	04/07/07
MK8	*Dickeya solani*	River 2	Scotland	03/09/07
MK9	*Duc3*	River 1	Scotland	04/07/07
MK10	*D. solani*	*Solanum tuberosum*, rotten tuber.	Israel	05/04/06
MK11	*D. solani*	*Solanum tuberosum*, asymptomatic tuber	Import	27/04/06
MK12	*D. solani*	*Solanum tuberosum*, asymptomatic tuber	Import	27/04/06
MK13	*D. solani*	*Solanum tuberosum*, rotten tuber.	Israel	14/05/07
MK14	*D. solani*	River 2	Scotland	21/07/08
MK15	*D. solani*	River 2	Scotland	30/07/08
MK16	*D. solani*	River 2	Scotland	30/07/08
MK17	*D. zeae*	*Zea mays*	USA	27/6/91
MK18	*D. zeae*	*Dieffenbachia picta*	Italy	2/3/92
MK19	*D. zeae*	River 3	Scotland	28/08/08

Strain	Attributed Species	Host isolated from	Country of isolation	Date of isolation
MK20	*D. zeae*	River 3	Scotland	7/10/08
CSL20710504	*D. solani*	*Solanum tuberosum* cv. Markies	England	2007
CSL20708100	*D. dianthicola.*	*Dahlia* sp.	England	2007
CSL20714261	*Duc2†*	*Phalaenopsis* sp.	England	2007
CSL20621674	*D. solani.*	*Hyacinthus* sp.	England	2007
CSL20714521	*D. dianthicola*	*Sedum* sp.	England	2007
A101/9	*D. solani*	*Solanum tuberosum*	Poland	
A101/10	*D. solani*	*Solanum tuberosum*	Poland	
A101/11	*D. solani*	*Solanum tuberosum*	Poland	
A101/12	*D. solani*	*Solanum tuberosum*	Poland	
B2744	*D. solani*	*Solanum tuberosum*	Scotland	2009
B2745	*D. solani*	*Solanum tuberosum*	Scotland	2009
B1	*D. solani*	*Solanum tuberosum, tubers*	Spain	2009
DM157	*D. solani*	*Solanum tuberosum* cv. Agria	Scotland (Dutch seed)	2009
DM159	*D. solani*	*Solanum tuberosum* cv. Agria	Scotland (Dutch seed)	2009
RW 192/1	*D. zeae phylotype II*	River water	England	

Strain	Attributed Species	Host isolated from	Country of isolation	Date of isolation
RW 240/1	*New D. sp.*	River water	England	
312	*Pectobacterium carotovorum*	*Solanum tuberosum*	Denmark	1952
402	*D. chrysanthemi*	*Chrysanthemum morifolium*	USA	1958
453	*D. dianthicola*	*Dianthus caryophyllus*	England,	1956
454	*D. dadantii*	*Philodendron sp.*	USA	1957
516	*D. chrysanthemi*	*Chrysanthemum sp.*		1957
549	*P. atrosepticum*	*Solanum tuberosum*	UK	1958
569	*D. sp.*	*Saccharum officinarum*	Australia	1958
898	*D. dadantii*	*Pelargonium capitatum*	Comoro Island	1960
1092	*Erwinia chrysanthemi*			
1121	*D. zeae*	*Ananas comosus*	Malaysia	1962
1385	*D. dianthicola*	*Dahlia sp.*	Romania	1962
1578	*P. rhapontici*	*Rheum rhaponticum*	England	1963
1861	*D. chrysanthemi*	*Parthenium argentatum*	USA	1966
1863	*D. zeae*	*Zea mays*	USA	1966
2260	*D. dianthicola*			
2264	*Erwinia chrysanthemi*	*Solanum tuberosum*	England	1995
2265	*E. chrysanthemi*			

Strain	Attributed Species	Host isolated from	Country of isolation	Date of isolation
2295	*Pantoea stewartii subsp. stewartii*		USA	1970
2339	*D. zeae*	*Chrysanthemum morifolium*	England	1970
2511	*D. paradisiaca*	*Musa paradisiaca var. dominico*	Colombia	1973
2538	*D. zeae*	*Zea mays*	USA	1973
2541	*D. zeae*	*Zea mays*	USA	1973
2546	*D. zeae*	*Zea mays*	India	1973
2795	*P. betavasculorum*	*Beta vulgaris*	USA	1975
2971	*E. herbicola*		Canada	1977
2976	*D. dieffenbachiae*	*Dieffenbachia sp.*	USA	1977
3004	*E. cypripedii*	*Cypripedium sp.*	USA	1977
3274	*Duc3*	*Agloanema sp.*	St. Lucia	1983
3531	*D. zeae*	*Solanum tuberosum*	Australia	1987
3532	*D. zeae*	*Solanum tuberosum*	Australia	1987
3533	*D. chrysanthemi*	*Solanum tuberosum*	USA	1987
3534	*D. dianthicola*	*Solanum tuberosum*	Netherlands	1987
3701	*P. wasabiae*	*Eutrema wasabi*	Japan	1990
3839	*P. carotovorum subsp. odoriferum*	*Cichorium intybus*	France	1992

Strain	Attributed Species	Host isolated from	Country of isolation	Date of isolation
6395	*P. chrysanthemi*	*Solanum tuberosum*	England	2006
6396	*P. chrysanthemi*	*Solanum tuberosum*	England	2006
6397	*P. chrysanthemi*	*Solanum tuberosum*	England	2006

Unless otherwise indicated, numbers refer to NCCPB (The National Collection of Plant Pathogenic Bacteria, Fera, England) assigned numbers.

MK refers to strains isolated at SASA (Kowalewska *et al*., 2010)

DM = *Dickeya* monitoring at SASA

CSL – Central Science Laboratory, Fera, England

RW = River Water survey at SASA

*DUC3 and †DUC2 as defined by Parkinson *et al*, 2009

Table A.2. List of EUPHRESCO Strains.

Strain	Attributed Species	Host isolated from	Country of isolation	Date of isolation
980	*Dickeya dianthicola*	*Solanum tuberosum*	Netherlands	
1259	*D. dieffenbachiae*	*Solanum tuberosum*	Hungary	
2019	*D. solani*	Hyacinthus sp.		
2114	*D. dianthicola*	*Dianthus caryophyllus*		
2115	*D. dianthicola*	*Dahlia sp.*		
2116	*D. dianthicola*	*Solanum tuberosum*	France	1975
2117	*D. chrysanthemi*	*Parthenium argentatum*		
2118 (402)	*D. chrysanthemi* pv. *chrysanthemi*	*Chrysanthemum morifolium*	USA	1956
2119	*D. chrysanthemi biovar chrysanthemi*	*Helianthus annuus*	France	1986
2120	*D. dadantii*	*Pelargonium capitatum*		
2121 (1121)	*D. dadantii*	*Ananas comosus*	Malaysia	1961
2122	*D. dadantii*	*Ipomea batatas*	Cuba	1987
2124	*D. dieffenbachiae*	*Dieffenbachiae sp.*	France	1970
2125 (2976)	*D. dieffenbachiae*	*Dieffenbachiae sp.*	USA	1957
2126	*D. dieffenbachiae*	*Solanum lycopersicum*	Cuba	1987
2127	*D. paradisiaca*	*Musa paradisiaca*	Colombia	1968
2129 (2511)	*D. paradisiaca*	*Musa paradisiaca var. dominico*	Colombia	1970

Strain	Attributed Species	Host isolated from	Country of isolation	Date of isolation
2131 (2538)	*D. zeae*	*Zea mays*	USA	1970
2132 (2339)	*D. zeae*	*Chrysanthemum morifolium*	UK	1970
2133	*D. zeae*	*Ananas comosus*	Martinique	1991
2187	*D. solani*	*Solanum tuberosum*	Israel	2006
2222	*D. solani*	*Solanum tuberosum*	Netherlands	2007
2276	*D. solani*	*Solanum tuberosum*	Poland	2005
3228	*D. solani*	*Solanum tuberosum*	Israel	
3239	*D. solani*	*Solanum tuberosum*	UK	
3294	*D. solani*	*Solanum tuberosum*	Finland	
3295	*D. solani*	*Solanum tuberosum*	Finland	
3296	*D. solani*	*Solanum tuberosum*	Israel	
3327 (3528)	*D. dianthicola*	*Solanum tuberosum*	UK	
3328 (3530)	*D. dianthicola*	*Solanum tuberosum*	UK	
3329 (3531)	*D. zeae*	*Solanum tuberosum*	Australia	
3330 (3533)	*D. chrysanthemi*	*Solanum tuberosum*	USA	
3332 (3237)	*D. dadantii*	*Solanum tuberosum*	Peru	
3334 (3344)	*D. dianthicola*	*Solanum tuberosum*	France	

Strain	Attributed Species	Host isolated from	Country of isolation	Date of isolation
3336	*D. solani*	*Solanum tuberosum*	France	
3337	*D. solani*	*Solanum tuberosum*	France	

Unless otherwise indicated, numbers refer to NCCPB (The National Collection of Plant Pathogenic Bacteria, Fera, England) assigned numbers.

MK refers to strains isolated at SASA (Kowalewska *et al*., 2010)

DM = *Dickeya* monitoring at SASA

CSL – Central Science Laboratory, Fera, England

RW = River Water survey at SASA

*DUC3 and †DUC2 as defined by Parkinson *et al*, 2009

Appendix 2. Materials and Methods Frequently Used.

A2.1 *Growth and Isolation of Bacterial Strains*

Throughout this research project a collection of strains has been consistently used. The details of which, including where and when they were isolated, is included Tables A1 and A2. The strains were isolated from environmental samples and sourced from a variety of collections. Once purified, cultures were stored at -80ºC (New Brunswick Scientific Ultra Low Temperature Freezer) on freezerbeads (Fisher Scientific) according to manufacturers' instructions and re-isolated as required onto Nutrient Agar and/or crystal-violet pectate medium (CVPM). The recipes for all media and solutions used can be found at the end of this chapter. In most cases, strains were isolated from freezerbeads, using at least two, onto Nutrient agar and re-isolated onto CVPM to ensure the presence of pit-forming colonies. Strains were grown at either 36ºC (*Dickeya* sp. and *Pectobacterium carotovorum* subsp. *carotovorum*) or 25ºC (*Pectobacterium atrosepticum*) for either 24 hours if on Nutrient Agar or 48 hours on CVPM in a chest incubator (Thermo Scientific HeraTherma incubator). All re-isolations using freezerbeads were carried out under aseptic conditions in a horizontal laminar flow hood (SLEE medical GmbH). Prior to use in experiments, the identity of strains were confirmed through conventional PCR.

A2.2 *Purification and Enrichment of Bacterial Isolates*

Dickeya and *Pectobacterium* spp. can exist in small numbers within environmental samples; therefore, when isolating from environmental samples it was necessary to include an enrichment step. Environmental samples were bashed using a plastic mallet to expose the inner material, added to Pectate Enrichment Media (PEM) and incubated at both 36ºC and 25ºC for 48 hours. PEM was sieved through muslin and centrifuged at 14,000rpm for 10 minutes to separate the bacteria. The pellet was resuspended in Ringer's Solution (Sigma) and diluted to 10^{-6} CFU.ml^{-1}. One hundred microliters of the suspension was plated onto CVPM and incubated at 36ºC and 25ºC for 48 hours. Pit-forming colonies were re-isolated onto Nutrient Agar and incubated at the appropriate temperature for 24 hours.

A2.3 *Preparation of Samples for PCR*

For identification of *Dickeya* spp., cultures were tested by PCR using the "Nassar assay" (Nassar *et al.*, 1996). A loopful of overnight Nutrient Agar culture was diluted in 500 µl of sterile distilled water (Sigma Aldrich) in a sterile 1.5 ml Eppendorf tube and vortexed. Once vortexed, the sample was boiled at 100ºC for 5 minutes (Grant Hotblock QBT2). Once cooled to room temperature and centrifuged briefly, the samples were either tested immediately or stored at -20ºC until required.

A2.4 *DNA Extraction*

DNA was also extracted from suspensions of boiled cells, as described above, using a method adapted from Pastrik & Maiss (2000) using Invitrogen "Easy DNA" extraction kit (Life Technologies, Paisley, UK). To 100 µl of the boiled sample (prepared as described in A2.3), 220 µl of lysis buffer was added and heated for 10 minutes at 95ºC before being placed on ice for 5 minutes. Once chilled, 80 µl lysozyme stock solution was then added and the samples incubated at 37ºC for 30 minutes. The samples were then mixed by vortexing with 220 µl of Easy DNA solution A (Invitrogen) and incubated at 65ºC for 30 minutes. After 30 minutes, 100 µl of Easy DNA solution B (Invitrogen) and 500 µl of chloroform was added. The samples were centrifuged at 15000*g* for 20 minutes at 4ºC. The upper phase was retained, washed with 1 ml of 100% ethanol, which had been stored at -20ºC, and incubated on ice for 10 minutes. The ethanol was removed by centrifuging at 15000*g* for 20 minutes at 4ºC. The pellet was washed with 500 µl 80% ethanol (aq., v/v) which had also been stored at -20ºC, and was again centrifuged at 15000*g* and 4ºC but for 10 minutes. The ethanol was removed and the pellet allowed to air dry at room temperature for at least 45 minutes. The pellet was resuspended in 100 µl sterile molecular grade water (Sigma Aldrich) and left at room temperature for at least 20 minutes before being stored at -20ºC until required.

A2.5 *Conventional PCR for Detection of* **Dickeya** *sp. – "Nassar Assay"*

Dickeya spp. were identified from extracted DNA or boiled cells suspensions using the conventional PCR assay of (Nassar *et al*., 1996). Primers (Table A.3) were synthesised by MWG (Eurofins, Germany) and JumpStart ReadyMix (Sigma) used throughout. Reactions (Table A.4) containing 1 µl of template were established in 96-well plates

(Sigma). PCR was conducted using a Veriti® Thermal Cycler (Life Technologies) and the temperature programme detailed in Table A.5.

Table A.3 Primers Used in Nassar Assay for Detection of *Dickeya* spp.

ADE 1 (Forward)	5'- GATCAGAAAGCCCGCAGCCAGAT-3'
ADE 2 (Reverse)	5'-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3'

Table A.4 PCR Reaction Mix for Nassar Assay

Component	Volume per Reaction (µl)
JumpStart™ REDTaq® ReadyMix™ Reaction Mix (Sigma)	12.5
ADE1 (25 pmol)	1
ADE2 (25 pmol)	1
Template	1l
Sterile H_2O (Sigma)	9.5
Final volume	**25**

Table A.5 PCR Cycles for Nassar Assay

94°C	1 min	x 25 cycles
72°C	2 min	
72°C	7 min	
4°C	∞	

A2.6 *Gel Electrophoresis of PCR Products*

A gel containing 80ml of TAE with 1% agarose was microwaved until all agarose had dissolved. The gel was cooled and 4µl of Gel Red (BioRad) added. Gels were poured into an electrophoresis tray (BioRad) containing a sample comb and allowed to solidify at room temperature. The comb was removed and the tank was flooded with TAE until the gel was covered. PCR products were pipetted into wells alongside 2 kb PCR marker (Sigma). Electrophoresis was carried out at a constant voltage of 80mV for at least 30 minutes. The migration of the PCR product was visualised by exposure to UV light. Images were captured using a BioDoc-It® Imaging System (UVP).

A2.7 *Preparation of Media*

All growth media were made as below, by Media Preparation Unit, Diagnostics and Molecular Biology Section, SASA and ingredients are per litre unless otherwise indicated.

Crystal-Violet Pectate Medium (CVPM) (based on Bdliya, 1995)

Table A.6 Basal Layer of CVPM

Peptone	2.0g
Yeast extract	0.6g
NaCl	1.0g
Agar	3.0g
Tryptone	1.5g
L-Asparagine	0.5g
SDS	50mg
Distilled water	250ml

Table A.7 Over Layer of CVPM

Crystal violet solution	1.0ml (0.075% w/v)
KNO_3	1.0g
$Na_3C_6H_5O_7$	2.5g
Agar	2.0g
Tryptone	1.5g
$CaCl_2$ .H2O	6.8ml (10% solution)
Bromothymol blue	0.5ml (0.5% solution)
Cold distilled water	500 ml
Sodium polypectate	9g

The semi-selective medium (CVPM) is for the isolation of pectolytic *Erwinia* sp. and consists of two layers:

0.5ml of sterilized 1% solution of 2,3,5-triphenyltetrazolium chloride was added to the basal medium (Table A.6) after autoclaving and cooled to 50^{o}C and 3.2 µl/ml Polymyxin B sulphate solution added. 12ml of the basal medium was dispensed per plate and allowed to set for 10 minutes.

The overlay was mixed as described in Table A.7 and autoclaved. Once cooled to 45^{o}C, 1.2 µg/ml of Polymyxin B sulphite was added. 15 ml of this layer was added on top of the basal layer.

Double Strength Pectate Enrichment Broth (PEM)

Table A.8 Ingredients of PEM per Litre

$MgSO_4$	0.64 g
$(NH_4)_2SO_4$	2.16 g
K_2HPO_4	2.16 g

The products as described in Table A.8 were dissolved separately into 300 ml dH_2O, added together in the order written and the solution made up to one litre using dH_2O. Into 5 ml of absolute ethanol, 3.4 g Sodium polypectate was suspended and then added to the solution and mixed well using a magnetic stirrer. The solution was steamed to ensure the polypectate was completely dissolved. The pH was adjusted to 7.2 as necessary. The entire solution was autoclaved for 15 minutes at 121^{o}C and stored at 4^{o}C. Once opened it was not re-used to avoid contamination.

Extraction Buffer

Table A.9 Extraction Buffer Components

di-Sodium Hydrogen Orthophosphate	4.26g
Potassium di-Hydrogen Orthophosphate	2.72g
Sodium Pyrophosphate Tetrabasic	1.0g

Lysis buffer

Table A.10 Components of Lysis Buffer

NaCl	100Mm
Tris-HCl (pH8.0)	10Mm
EDTA (pH8.0)	1mM EDTA

Lysozyme Stock Solution

50mg lysozyme from chicken egg white (Sigma-Aldrich) was added to 1 ml of 10 mM Tris-HCl (pH 8.0).

Nutrient Agar

To one litre of dH_2O, 28 g of Nutrient agar (Sigma Aldrich) was added, then autoclaved for 15 minutes at 121°C and cooled to 45°C before dispensing 20 ml per plate.

TE Buffer

Table A.11 Components of TE Buffer

Tris-HCl (pH8.0)	10Mm
EDTA	1Mm

Appendix 3. Daily Maximum and Minimum Temperatures during Growing Seasons 2010-2012

Table A.12 The Daily Maximum and Minimum Temperatures (ºC) Measured by the Met Office for Gogarbank, Edinburgh (Met Office station 03166) between April and October of the Three Growing Seasons.

DATE	DAILY MAX 2010 (ºC)	DAILY MAX 2011 (ºC)	DAILY MAX 2012 (ºC)	DATE	DAILY MIN 2010 (ºC)	DAILY MIN 2011 (ºC)	DAILY MIN 2012 (ºC)
01-Apr	8.1	14.8	14.6	01-Apr	0.6	8	-0.8
02-Apr	9.1	13	10	02-Apr	-2.2	8.3	7.4
03-Apr	9.8	12.4	5.1	03-Apr	3.9	6.9	1.3
04-Apr	9.3	13.6	6.8	04-Apr	3.7	5.2	1.3
05-Apr	11.7	16.1	10.5	05-Apr	4.2	6.7	-3.8
06-Apr	11.7	17.7	10.9	06-Apr	8.8	13.4	4.1
07-Apr	12.2	12.9	12	07-Apr	4	7.6	4.5
08-Apr	12.5	16.8	12.3	08-Apr	6	7.2	5.7
09-Apr	14.7	17.2	10.9	09-Apr	5.1	5.5	5.5
10-Apr	18.4	21.5	10.6	10-Apr	2.4	6.5	0.8
11-Apr	15.4	13.6	12.5	11-Apr	3	11.9	1
12-Apr	15.7	13.1	10.6	12-Apr	3.7	4.4	4.6
13-Apr	12.1	12	9.7	13-Apr	6.4	5.9	1.1
14-Apr	9.9	13.8	9.4	14-Apr	5.9	8.6	0.6
15-Apr	14.6	15.3	9.2	15-Apr	3.2	7.5	-0.7
16-Apr	16.2	12.3	10.3	16-Apr	0.8	8.4	-1.5
17-Apr	12.5	17.5	11.9	17-Apr	5.5	5.5	4.5
18-Apr	7.3	15.2	10.8	18-Apr	4.3	4.2	2
19-Apr	10.3	19	9.1	19-Apr	1.9	6.8	5.3

DATE	DAILY MAX 2010 (ºC)	DAILY MAX 2011 (ºC)	DAILY MAX 2012 (ºC)	DATE	DAILY MIN 2010 (ºC)	DAILY MIN 2011 (ºC)	DAILY MIN 2012 (ºC)
20-Apr	10.9	16.8	11.9	20-Apr	3.6	4.6	5.2
21-Apr	12.4	16.7	11.9	21-Apr	-0.1	6.1	5.6
22-Apr	12.1	12.7	11.6	22-Apr	0.3	6.1	4.3
23-Apr	10.5	13	10.3	23-Apr	6.7	7.3	5.6
24-Apr	14.9	16.5	10.1	24-Apr	6.1	2	5.5
25-Apr	15.5	15.4	8.3	25-Apr	7.2	7.5	4.8
26-Apr	15.5	13.9	9.9	26-Apr	8.6	2.1	6.4
27-Apr	16	13.7	10.6	27-Apr	7	-0.1	0.8
28-Apr	16.7	14.8	9.1	28-Apr	10.1	0.9	3.3
29-Apr	15.6	13.5	7.4	29-Apr	11.1	4.2	1.4
30-Apr	14.6	17.4	8.9	30-Apr	6.7	7.7	4.8
01-May	10.1	15.3	10.6	01-May	7	2.3	6.8
02-May	9.4	16.7	10.1	02-May	5	3.4	6.4
03-May	12	12.8	13.5	03-May	-0.9	0.1	5.4
04-May	13.4	16.6	10.2	04-May	3.3	-0.8	6.2
05-May	16.4	14	10.7	05-May	9	7.2	-1.8
06-May	12	18.1	9.8	06-May	8.4	10.2	1
07-May	11.7	20.4	10	07-May	5.8	11.4	0.2
08-May	11.7	16.7	13.8	08-May	-0.1	10.3	2.4
09-May	14.4	18	13.5	09-May	2.5	6.9	3.5
10-May	10.9	16.5	7	10-May	4.4	9.8	5.4
11-May	10.8	15.2	9.9	11-May	-0.4	9.2	3.9
12-May	10.8	14.7	14.2	12-May	1.4	8.2	2.6
13-May	11.9	15.1	11.4	13-May	2.2	6.8	6.5

DATE	DAILY MAX 2010 (ºC)	DAILY MAX 2011 (ºC)	DAILY MAX 2012 (ºC)	DATE	DAILY MIN 2010 (ºC)	DAILY MIN 2011 (ºC)	DAILY MIN 2012 (ºC)
14-May	14.1	15	12.1	14-May	5.9	7	4.5
15-May	14.4	14.1	13.1	15-May	5.7	7.1	5
16-May	14.8	14.4	11.6	16-May	7.3	9.2	2.1
17-May	16.8	15.2	10	17-May	6.5	8.2	6.7
18-May	19.1	13.2	7.3	18-May	5.1	9	5.5
19-May	17.2	15.1	9.8	19-May	8	7	5.3
20-May	21	14.1	12.5	20-May	9	6.3	1.3
21-May	22.9	15.3	14.5	21-May	10.3	7.5	5.7
22-May	25.2	14	19.1	22-May	12.5	7.1	7.5
23-May	25.2	13.8	21	23-May	11.8	7.8	6.8
24-May	18.2	12.6	23.4	24-May	10.4	6.6	11.4
25-May	15	13.2	18.7	25-May	7.3	5.8	11
26-May	13.9	14.2	20.5	26-May	6.4	7.4	7.2
27-May	13.2	13.4	21.4	27-May	5	7.2	11.1
28-May	13.9	13.4	22.2	28-May	3.1	8.5	7.9
29-May	14.5	14.5	16.8	29-May	6	7.5	9.6
30-May	13.8	15.3	12.8	30-May	7.7	6.9	10
31-May	16	16.3	15	31-May	2.2	4.4	11
01-Jun	15.5	17.7	16.8	01-Jun	11	8.7	8.7
02-Jun	18.4	20.2	11.7	02-Jun	9.1	12.4	8.6
03-Jun	21.1	25.7	12.6	03-Jun	6.3	8.3	3.6
04-Jun	19.8	12.5	16.1	04-Jun	10.4	10.3	2.9
05-Jun	23.3	12.2	15.8	05-Jun	8.3	8.7	5.6
06-Jun	12.7	15.1	14	06-Jun	11	7.5	10.2

DATE	DAILY MAX 2010 (ºC)	DAILY MAX 2011 (ºC)	DAILY MAX 2012 (ºC)	DATE	DAILY MIN 2010 (ºC)	DAILY MIN 2011 (ºC)	DAILY MIN 2012 (ºC)
07-Jun	14	15.3	15.3	07-Jun	11.3	8.2	6.4
08-Jun	12.7	16.9	15.3	08-Jun	11.3	5.2	11
09-Jun	13.8	14.6	18.2	09-Jun	10.3	7.9	11.1
10-Jun	14.7	15.2	13.3	10-Jun	10.3	4.3	10.5
11-Jun	18.4	13.2	13.5	11-Jun	7.5	4.4	8.7
12-Jun	17	15.3	14.7	12-Jun	8	6.6	5.5
13-Jun	16.2	16.1	16	13-Jun	9.8	9.1	7.5
14-Jun	15.7	18.5	16.6	14-Jun	10	9.1	7.8
15-Jun	19.6	19.8	9.9	15-Jun	6.3	11	8.8
16-Jun	21.7	17.7	10.6	16-Jun	13.2	10.6	8.8
17-Jun	24.4	15.5	12.8	17-Jun	14.6	7.9	8.8
18-Jun	18.7	13.5	18.1	18-Jun	12.8	7.6	4.3
19-Jun	17.5	16.4	18.4	19-Jun	5.3	10.5	9
20-Jun	23.2	18.5	17.5	20-Jun	3.9	9.4	5.3
21-Jun	21.7	13.1	17	21-Jun	8	10	10.5
22-Jun	23.5	16.2	17.5	22-Jun	9.6	10.3	11
23-Jun	20.6	17.8	15	23-Jun	11.3	9.3	10.8
24-Jun	19.2	17	18.2	24-Jun	11.3	7.1	10.7
25-Jun	21.7	19.2	19.6	25-Jun	9.1	9.5	9.1
26-Jun	23.8	21.1	18.7	26-Jun	11.5	12.7	7.8
27-Jun	22.8	14.8	16.9	27-Jun	12.2	13.3	12
28-Jun	20.2	19.3	21.8	28-Jun	13.6	8.3	12.5
29-Jun	22	17.9	17.9	29-Jun	14.2	7.7	13.3
30-Jun	23.9	17.3	18.9	30-Jun	10.3	8.2	12

DATE	DAILY MAX 2010 (ºC)	DAILY MAX 2011 (ºC)	DAILY MAX 2012 (ºC)	DATE	DAILY MIN 2010 (ºC)	DAILY MIN 2011 (ºC)	DAILY MIN 2012 (ºC)
01-Jul	21.5	19.1	17.4	01-Jul	14.3	8	10.4
02-Jul	21.3	19.9	17.9	02-Jul	13.1	5.4	10.6
03-Jul	19.6	23	19.8	03-Jul	12	6.5	12.8
04-Jul	20	21.6	21	04-Jul	13.6	8.9	13.9
05-Jul	17.3	21.8	17.6	05-Jul	11.4	14.5	13.4
06-Jul	17.4	17.7	16.5	06-Jul	10	12.7	13.3
07-Jul	19.2	18.7	13.1	07-Jul	13.1	9.1	12.3
08-Jul	19.9	17.6	15.6	08-Jul	11.6	6.4	11.2
09-Jul	20.4	20.2	13.4	09-Jul	12.3	10.5	12.4
10-Jul	17.5	19.5	13.9	10-Jul	10.5	10.9	10.7
11-Jul	17.4	17.3	13.9	11-Jul	12.4	10.6	10.6
12-Jul	19.7	17.6	15.2	12-Jul	9.8	11.5	9.6
13-Jul	19.2	18.8	14.2	13-Jul	7.3	7.5	10.2
14-Jul	15.4	22.9	16	14-Jul	11.4	7.7	9.1
15-Jul	18.6	21.2	16.5	15-Jul	11.5	11.5	8.5
16-Jul	16.5	18.7	18	16-Jul	11.8	13.9	9.5
17-Jul	18.5	19	17.4	17-Jul	11.7	12.6	9.1
18-Jul	22	17.5	15.3	18-Jul	12.1	10.6	12.2
19-Jul	19.3	18.7	16	19-Jul	14.9	11.4	9.8
20-Jul	23.5	14.7	17.4	20-Jul	13.1	11.2	7.2
21-Jul	16.6	17.1	18.2	21-Jul	14.3	8.5	7.8
22-Jul	16.8	16.3	20.8	22-Jul	10.6	10.1	13.5
23-Jul	20.5	19.5	17	23-Jul	8.8	6.1	15.9
24-Jul	19.3	21.2	20.8	24-Jul	6.8	5.5	13.1

DATE	DAILY MAX 2010 (ºC)	DAILY MAX 2011 (ºC)	DAILY MAX 2012 (ºC)	DATE	DAILY MIN 2010 (ºC)	DAILY MIN 2011 (ºC)	DAILY MIN 2012 (ºC)
25-Jul	20	20.1	20.3	25-Jul	13.2	8.2	10.5
26-Jul	20.1	19.2	19.6	26-Jul	10.7	8.7	10.1
27-Jul	19.1	21.5	17.9	27-Jul	13.4	6.5	10.7
28-Jul	19.9	16.3	16.9	28-Jul	11.9	11.2	10.6
29-Jul	20.2	20.8	17.2	29-Jul	11.9	10.7	9.9
30-Jul	18.9	20.5	16.2	30-Jul	10.6	9.2	9.3
31-Jul	18.5	19.6	18.4	31-Jul	12.5	9.8	7.4
01-Aug	19.7	19.9	19.3	01-Aug	12.4	16.2	12.4
02-Aug	17.9	19	19.7	02-Aug	12.2	14.1	9.8
03-Aug	19	22.1	18.8	03-Aug	12.1	11.5	7.3
04-Aug	19.8	21	20.5	04-Aug	11.6	12.3	11.7
05-Aug	19.5	19.7	15.4	05-Aug	9.9	14	13.8
06-Aug	20.3	17.6	19.2	06-Aug	10.3	8.8	12.7
07-Aug	21.7	16.7	19.9	07-Aug	13.7	10.7	10.1
08-Aug	21.3	19.7	19.9	08-Aug	13.3	10.1	7.4
09-Aug	20.4	18.8	21.5	09-Aug	12.1	8.5	7
10-Aug	17.3	18	20.5	10-Aug	8.5	10	10.2
11-Aug	18.5	13.8	20	11-Aug	9.8	11.6	8.1
12-Aug	17.8	17.8	21.5	12-Aug	11.8	11.6	12.2
13-Aug	20.3	19.2	21.5	13-Aug	10.9	13.8	14.6
14-Aug	19.2	19.2	22.6	14-Aug	11	12.5	12.9
15-Aug	22.1	18.2	20.7	15-Aug	8.3	9.4	10.7
16-Aug	17.3	16.3	21	16-Aug	7.5	10.8	13.2
17-Aug	20.7	17.9	21.4	17-Aug	13	8.5	13.9

DATE	DAILY MAX 2010 (ºC)	DAILY MAX 2011 (ºC)	DAILY MAX 2012 (ºC)	DATE	DAILY MIN 2010 (ºC)	DAILY MIN 2011 (ºC)	DAILY MIN 2012 (ºC)
18-Aug	18.4	15.5	21.8	18-Aug	8.3	10.4	16
19-Aug	19.8	17.7	20.8	19-Aug	7.8	9.7	10.2
20-Aug	23.1	20.1	22	20-Aug	12.7	11.2	9.3
21-Aug	19.9	19.5	20.3	21-Aug	12.7	10.9	11.5
22-Aug	19.2	17.9	19.2	22-Aug	10.5	10.3	12
23-Aug	14.6	18.9	18.6	23-Aug	8.7	6.9	11.9
24-Aug	16.2	18.1	18.3	24-Aug	10.9	11.4	12.5
25-Aug	18.8	18.2	16.3	25-Aug	9.2	7.3	12.3
26-Aug	17.6	16.5	16.2	26-Aug	7.9	8.4	10.3
27-Aug	19.1	17.4	17.1	27-Aug	7.1	10.9	9.7
28-Aug	17.8	13.6	18.9	28-Aug	9.8	9	11.9
29-Aug	16.1	16.6	19.3	29-Aug	11.1	8.5	12.4
30-Aug	17.9	16.4	15.1	30-Aug	3.8	10.7	7.2
31-Aug	19.5	15.2	15.4	31-Aug	3.6	9.9	3.7
01-Sep	18.4	16.9	19.1	01-Sep	5.4	10.6	10.7
02-Sep	18.5	18.7	19.6	02-Sep	11.7	12.5	12.7
03-Sep	21.5	19.9	22	03-Sep	8.3	12.4	13.9
04-Sep	21.9	18.6	18.2	04-Sep	9.7	6.3	12.2
05-Sep	19.9	17.5	18.5	05-Sep	13.2	6.9	10.3
06-Sep	19.6	16	17.3	06-Sep	13.9	11.5	9.2
07-Sep	18	16.9	19.5	07-Sep	11.8	10.4	13.7
08-Sep	20.4	17.2	19.1	08-Sep	9.1	9.7	15
09-Sep	19.8	19.3	18.3	09-Sep	11	10	5.6
10-Sep	19.3	21.2	14.9	10-Sep	11.9	11	13.4

DATE	DAILY MAX 2010 (ºC)	DAILY MAX 2011 (ºC)	DAILY MAX 2012 (ºC)	DATE	DAILY MIN 2010 (ºC)	DAILY MIN 2011 (ºC)	DAILY MIN 2012 (ºC)
11-Sep	17.9	17	13.5	11-Sep	10.5	12.5	7
12-Sep	18.5	16.6	15.4	12-Sep	11	12.1	8.4
13-Sep	18.2	13.9	15.5	13-Sep	12.4	10.7	6.3
14-Sep	15.1	15.1	16.3	14-Sep	12.5	9.7	10.2
15-Sep	17.3	15.2	16.1	15-Sep	8	3.6	10.8
16-Sep	16.1	14.1	16.2	16-Sep	8.6	6.3	11.9
17-Sep	15.7	15.5	15.4	17-Sep	6.4	10.3	9.2
18-Sep	16.3	14.6	14	18-Sep	8.7	7.9	6.8
19-Sep	15.4	15.5	14.9	19-Sep	10.3	7.3	5.3
20-Sep	18.3	15.2	10.7	20-Sep	11.4	9.9	8.2
21-Sep	19.2	13	13.3	21-Sep	8.9	10.3	3.5
22-Sep	18	15	14.4	22-Sep	12.5	7.9	0.4
23-Sep	13.1	17.3	13.7	23-Sep	12.4	10.7	0.4
24-Sep	13.4	18.5	10.8	24-Sep	7.1	12.8	7.1
25-Sep	13.1	17.6	12	25-Sep	3.5	11.6	8.8
26-Sep	13.7	15.8	13.6	26-Sep	2.2	9.2	9.7
27-Sep	12.1	18.9	12.5	27-Sep	3.9	10.4	8.3
28-Sep	16	24	13.5	28-Sep	10.1	10.8	8.9
29-Sep	15.1	21.1	14.2	29-Sep	12.1	13.7	7.5
30-Sep	15.1	25	15.5	30-Sep	4.8	11.3	10.1
01-Oct	15	17.1	14.5	01-Oct	7.4	14.4	10
02-Oct	16	15.7	13.5	02-Oct	5.5	13.1	7.9
03-Oct	14.9	18.3	13.3	03-Oct	11	11.9	7
04-Oct	16.5	16.3	13.6	04-Oct	8.9	10.3	2.6

DATE	DAILY MAX 2010 (ºC)	DAILY MAX 2011 (ºC)	DAILY MAX 2012 (ºC)	DATE	DAILY MIN 2010 (ºC)	DAILY MIN 2011 (ºC)	DAILY MIN 2012 (ºC)
05-Oct	16.2	17.7	12.9	05-Oct	12	10.4	4.1
06-Oct	14.5	10.8	13.5	06-Oct	9	5.8	6.3
07-Oct	18.4	14.3	13.1	07-Oct	9.2	4.8	0.1
08-Oct	15.8	15.1	13.4	08-Oct	8.3	7.9	0.1
09-Oct	12.9	16.3	11.7	09-Oct	12.4	9.3	-0.3
10-Oct	12.2	12.5	12.9	10-Oct	11.3	10.8	-1
11-Oct	13.1	14.1	14.9	11-Oct	9.9	8.8	3
12-Oct	11.9	10.9	10.6	12-Oct	3.9	7.5	8.9
13-Oct	9.3	14.8	9.8	13-Oct	6.1	8.1	7.5
14-Oct	14.1	17.5	10.7	14-Oct	6.6	9.2	6.8
15-Oct	12	14.6	10.3	15-Oct	9.3	12.2	1.3
16-Oct	12.8	14.7	7.3	16-Oct	3.6	8.8	1.3
17-Oct	12.2	11	9.4	17-Oct	6.2	9.9	-0.9
18-Oct	11.3	10.1	11.8	18-Oct	9.4	4.2	3.2
19-Oct	9.5	9.1	9.8	19-Oct	4.3	3.8	8.2
20-Oct	8.9	13	12.9	20-Oct	-0.1	2.6	7.7
21-Oct	12.5	14.5	14	21-Oct	2.9	7.3	4.3
22-Oct	12.1	13.6	9.5	22-Oct	7.4	10.9	0.6
23-Oct	9.2	16.8	10.4	23-Oct	5.1	9.5	6.5
24-Oct	8.8	16.2	10.8	24-Oct	0.6	10.9	9.3
25-Oct	12.3	15	11.4	25-Oct	-2.5	11.1	5.6
26-Oct	15.6	12.7	6.4	26-Oct	3.4	5.1	1.8
27-Oct	13	12.4	9.2	27-Oct	10.3	6.2	-0.9
28-Oct	15.1	12.3	9.7	28-Oct	8	6.5	2.7

DATE	DAILY MAX 2010 (ºC)	DAILY MAX 2011 (ºC)	DAILY MAX 2012 (ºC)	DATE	DAILY MIN 2010 (ºC)	DAILY MIN 2011 (ºC)	DAILY MIN 2012 (ºC)
29-Oct	16	14	8.7	29-Oct	9.3	8.5	4
30-Oct	11.9	16.3	9	30-Oct	7	10.8	3.4
31-Oct	11.6	16.4	7.3	31-Oct	1.9	12.4	5.6

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